

BLEOMYCIN GENE CLUSTER COMPONENTS AND THEIR USES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit under 35 U.S.C. §119 of provisional applications USSN 60/115,435, filed on January 6, 1999, and USSN 60/118,848, filed on
5 February 5, 1999, both of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

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10 American Cancer Society and the School of Medicine, University of California, Davis, National Institutes of Health Grant Number A140475, and a grant from the Searle Scholars Program of the Chicago Community Trust. The Government of the United States of America may have certain rights in this invention.

FIELD OF THE INVENTION

15 This invention relates the field of polyketide synthesis and nonribosomal polypeptide synthesis. In particular this invention pertains to the isolation of the bleomycin gene cluster which encodes the first identified hybrid polyketide synthase/nonribosomal peptide synthetase pathway.

BACKGROUND OF THE INVENTION

20 Polyketides and nonribosomal peptides are two large families of natural products that include many clinically valuable drugs, such as erythromycin and vancomycin (antibacterial), FK506 and cyclosporin (immunosuppressant), and epothilone and bleomycin (BLM) (antitumor). The biosyntheses of polyketides and nonribosomal peptides are catalyzed by polyketide synthases (PKSs) (Hopwood (1997) *Chem. Rev.* 97: 2465; Katz
25 (1997) *Chem. Rev.*, 97: 2557; C. Khosla, (1997) *Chem. Rev.*, 97: 2577; Ikeda and Omura, (1997) *Chem. Rev.*, 97: 2591; Staunton and Wilkinson(1997) *Chem. Rev.*, 97: 2611; Cane *et al.*(1998) *Science* 282: 63) and nonribosomal peptide synthetases (NRPSs) (Cane *et al.*(1998) *Science* 282: 63. Marahiel *et al.* (1997) *Chem. Rev.* 97: 2651; von Döhren *et al.* (1997) *Chem. Rev.* 97: 2675), respectively. Remarkably, PKSs and NRPSs use a very

similar strategy for the assembly of these two distinct classes of natural products by sequential condensation of short carboxylic acids and amino acids, respectively, and utilize the same 4'-phosphopantetheine prosthetic group, via a thioester linkage, to channel the growing polyketide or peptide intermediate during the elongation processes.

Both type I PKSs and NRPSs are multifunctional proteins that are organized into modules. (A module is defined as a set of distinctive domains that encode all the enzyme activities necessary for one cycle of polyketide or peptide chain elongation and associated modifications.) The number and order of modules and the type of domains within a module on each PKS or NRPS protein determine the structural variations of the resulting polyketide and peptide products by dictating the number, order, choice of the carboxylic acid or amino acid to be incorporated, and the modifications associated with a particular cycle of elongation. These features of PKS and NRPS inspired us to search for a hybrid PKS and NRPS system. Since the modular architecture of both PKS (Cane *et al.* (1998) *Science* 282: 63; Katz and Danadio (1993) *Ann. Rev. Microbiol.* 47: 875 (1993); Hutchinson and Fujii (1995) *Ann. Rev. Microbiol.* 49: 201) and NRPS (Cane *et al.* (1998) *Science* 282: 63, Stachelhaus *et al.* (1995) *Science* 269: 69; Stachelhaus *et al.* (1998) *Mol. Gen. Genet.* 257: 308; Belshaw *et al.* (1999) *Science* 284, 486) has been exploited successfully in combinatorial biosynthesis of diverse "unnatural" natural products, it is imagined that a hybrid PKS and NRPS system, capable of incorporating both carboxylic acids and amino acids into the final products, could surely lead to even greater chemical structural diversity.

The BLMs, differing structurally at the C-terminal amines of the glycopeptides, are a family of antibiotics produced by *Streptomyces verticillus* (Sv). BLMs exhibit strong antitumor activity through a metal-dependent oxidative cleavage of DNA or RNA in the presence of molecular oxygen and are incorporated into current chemotherapy of several malignancies under the trade name of Bleomoxane[®] that contains BLM A2 and BLM B2 as the principal constituents (Sikic *et al.* Eds. (1985) *Bleomycin Chemotherapy*, Academic Press, New York; Natrajan and Hecht (1994) pages 197-242 In: *Molecular Aspects of Anticancer Drug-DNA Interaction Vol. 2*, Neidle and Waring Eds., Macmillan, London). Umezawa, Fujii, Takita, and co-workers extensively studied the biosynthesis of BLM in Sv ATCC15003 by feeding isotope-labeled precursors and by isolating various biosynthetic intermediates and shunt metabolites, establishing that the BLMs are in fact natural hybrid metabolites of polyketide and peptide biosynthesis (Takita and Muroka (1990) pages 289-309 In: *Biochemistry of Peptide Antibiotics: Recent Advances in the Biotechnology of β -Lactams and Microbial Peptides*, Kleinkauf and Von Döhren Eds., W. de

Gruyter, New York). On the assumption that BLM biosynthesis follows the paradigm for peptide and polyketide biosynthesis, we predict that the Blm megasynthetase, which catalyzes the assembly of the BLM backbone from nine amino acids and one acetate, should bear the characteristics of both NRPS and PKS, providing an excellent model to study the mechanism by which NRPS and PKS could be integrated into a productive biosynthetic system to synthesize a hybrid peptide and polyketide metabolite (Fig. 1A) (Shen *et al.* (1999) *Bioorg. Chem.* 27: 155).

SUMMARY OF THE INVENTION

This invention pertains to the isolation and elucidation of the bleomycin gene cluster. Nucleic acid sequences encoding all of the open reading frames (ORFs) that encode polypeptides sufficient to direct the biosynthesis of bleomycin are provided. The nucleic acids can be used in their "native" format or recombined in a wide variety of manners to create novel synthetic pathways.

See 22
In one embodiment, this invention provides an isolated nucleic acid comprising a nucleic acid selected from the group consisting of a nucleic acid encoding any one of Blm open reading frames (ORFs) 8 through 41, and/or a nucleic acid encoding a polypeptide encoded by any one of Blm open reading frames (ORFs) 8 through 41, and/or a nucleic acid amplified by polymerase chain reaction (PCR) using any one of the primer pairs identified in Table II and the nucleic acid of a bleomycin-producing organism as a template. The nucleic acid may comprise one or multiple (*e.g.* two, more preferably 3 or more) bleomycin open reading frames (*i.e.* BLM ORFs 8 through 41). One preferred nucleic acid comprises a nucleic acid encoding a C domain lacking one or more His residues of the conserved HHxxxDG active site for transpeptidation. In another preferred embodiment the nucleic acid comprises a nucleic acid encoding a protein encoded by a gene selected from the group consisting of blmI, blmII, and blmXI.

In another embodiment this invention provides an isolated nucleic acid encoding a (biosynthetic) module comprising two or more (more preferably three or more, most preferably four or more) catalytic domains of a protein encoded by a nucleic acid of a bleomycin gene cluster wherein said catalytic domains are selected from the group consisting of a condensation (C) domain, an adenylation (A) domain, a peptidyl carrier protein (PCP) domain, a condensation/cyclization domain (Cy), an acyl-carrier protein (ACP)-like domain, an oxidization domain (Ox), a ketoacyl synthase (KS) domain, an acetyl transferase (AT) domain, a ketoreductase (KR) domain, and a methyltransferase (MT) domain. Preferred

nucleic acids comprises a nucleic acid encoding one or more proteins comprising a module selected from the group consisting of NRPS-0, NRPS-1, NRPS-2, NRPS-3, NRPS-4, NRPS-5, NRPS-6, NRPS-7, NRPS-7, NRPS-9, and PKS. Particularly preferred nucleic acids comprise an open reading frame from SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

5 In still another embodiment, this invention provides an isolated nucleic acid comprising a nucleic acid encoding a protein encoded by a gene from a BLM gene cluster. Preferred nucleic acids encode a protein encoded by a gene selected from the group consisting of blmI, blmII, and blmXI. In another embodiment, preferred nucleic acids encode a protein encoded by a gene selected from the group consisting of blmIII, blmIV, 10 blmV, blmVI, blmVII, blmIX, and blmX. In still yet another embodiment, the nucleic acid comprises a nucleic acid encoding a protein encoded by blmVIII. Particularly preferred nucleic acids comprise a nucleic acid selected from the group consisting of blmI, blmII, and blmXI. Other particularly preferred nucleic acids comprise a nucleic acid selected from the group consisting of blmIII, blmIV, blmV, blmVI, blmVII, blmIX, and blmX, while still other 15 particularly preferred nucleic acids comprise blmVIII.

In still yet another embodiment, this invention provides an isolated nucleic acid comprising a nucleic acid that encodes a protein comprising at least one catalytic domain selected from the group consisting of a condensation (C) domain, an adenylation (A) domain, a peptidyl carrier protein (PCP) domain, a condensation/cyclization domain (Cy), an 20 acyl-carrier protein (ACP)-like domain, an oxidization domain (Ox), a ketoacyl synthase (KS) domain, an acetyl transferase (AT) domain, a ketoreductase (KR) domain, and a methyltransferase (MT) domain, and that hybridizes to a nucleic acid selected from the group consisting of orf8, orf9, orf10, orf11, orf12, orf13, orf14, orf15, orf15, orf16, orf17, orf18, orf19, orf20, orf21, orf22, orf23, orf24, orf25, orf26, orf27, orf28, orf29, orf30, orf31, orf32, 25 orf33, orf34, orf35, orf36, orf37, orf38, orf39, and orf40 under stringent conditions. In certain embodiments this also includes nucleic acids that would stringently hybridizes indicated above, but for, the degeneracy of the nucleic acid code. In other words, if silent mutations could be made in the subject sequence so that it hybridizes to the indicated sequence(s) under stringent conditions, it would be included in certain embodiments. A 30 preferred isolated nucleic acid comprises a nucleic acid encoding a module. A particularly preferred isolated nucleic acid comprises a nucleic acid encoding a BLM gene.

This invention also provides a nucleic acid comprising a nucleic acid selected from the group consisting of consisting of orf8, orf9, orf10, orf11, orf12, orf13, orf14, orf15, orf15, orf16, orf17, orf18, orf19, orf20, orf21, orf22, orf23, orf24, orf25, orf26, orf27, orf28,

orf29, orf30, orf31, orf32, orf33, orf34, orf35, orf36, orf37, orf38, orf39, and orf40, or an allelic variant thereof. Preferred nucleic acids comprise a nucleic acid that is a single nucleotide polymorphism (SNP) of a nucleic acid selected from the group consisting of consisting of orf8, orf9, orf10, orf11, orf12, orf13, orf14, orf15, orf15, orf16, orf17, orf18, 5 orf19, orf20, orf21, orf22, orf23, orf24, orf25, orf26, orf27, orf28, orf29, orf30, orf31, orf32, orf33, orf34, orf35, orf36, orf37, orf38, orf39, and orf40.

This invention also provides an isolated gene cluster comprising open reading frames encoding polypeptides sufficient to direct the assembly of a bleomycin.

In one embodiment this invention provides an isolated multi-functional 10 protein complex comprising both a polyketide synthase (PKS) and a polypeptide synthetase (NRPS) and/or an isolated nucleic acid encoding a multi-functional protein complex comprising both a polyketide synthase (PKS) and a polypeptide synthetase (NRPS).

This invention also provides various *blm* cluster polypeptides or *blm* cluster-derived polypeptides. Thus, in one embodiment this invention provides an isolated 15 polypeptide comprising a catalytic domain encoded by a nucleic acid of a bleomycin gene cluster wherein said nucleic acid comprises a nucleic acid selected from the group consisting of a nucleic acid encoding any one of Blm open reading frames (ORFs) 8 through 41; and/or a nucleic acid amplified by polymerase chain reaction (PCR) using any one of the primer pairs identified in Table II. Preferred polypeptides comprise an enzymatic domain selected 20 from the group consisting of a condensation (C) domain, an adenylation (A) domain, a peptidyl carrier protein (PCP) domain, a condensation/cyclization domain (Cy), an acyl-carrier protein (ACP)-like domain, an oxidization domain (Ox), a ketoacyl synthase (KS) domain, an acetyl transferase (AT) domain, a ketoreductase (KR) domain, and a methyltransferase (MT) domain. Particularly preferred polypeptides are encoded by the 25 nucleic acids described above and herein.

This invention also provides expression vectors comprising any of the nucleic acids described herein and/or host cells (*e.g.* *Streptomyces*) transfected and/or transformed with any of these expression vectors. A preferred host cell is transformed with an exogenous nucleic acid comprising a gene cluster encoding polypeptides sufficient to direct the 30 assembly of a bleomycin or bleomycin analog.

This invention also provides methods of use of the *blm* and *blm*-derived nucleic acid(s) and/or polypeptides. One such method is a method of chemically modifying a biological molecule. The method involves contacting a biological molecule that is a substrate for a polypeptide encoded by one or more bleomycin biosynthesis gene cluster

open reading frames with the polypeptide encoded by one or more bleomycin biosynthesis gene cluster open reading frames, whereby the polypeptide chemically modifies the biological molecule. In one particularly preferred embodiment, the biological molecule is an amino acid and said polypeptide is a peptide synthetase. In another preferred embodiment, the polypeptide is a methyl transferase. Other substrates and *blm* encoded polypeptides are illustrated in Table II.

In another embodiment this invention provides a method of coupling a first amino acid to a second amino acid. This method involves contacting the first and second amino acid with a recombinantly expressed bleomycin nonribosomal peptide synthetase (NRPS). A preferred NRPS is selected from the group consisting of NRPS-5, NRPS-4, NRPS-3, NRPS-9, NRPS-8, and NRPS-7. Another preferred NRPS is selected from the group consisting of NRPS-6, NRPS-2, NRPS-1, and NRPS-0. The contacting can be *in vivo* (e.g. in a host cell) or *ex vivo*.

In another embodiment this invention provides a methods of coupling a first fatty acid to a second fatty acid, said method comprising contacting the first and second fatty acids with a recombinantly expressed bleomycin polyketide synthase (PKS). Again, the contacting can be *in vivo* (e.g. in a host cell) or *ex vivo*.

In still another embodiment, this invention provides a method of producing a bleomycin or bleomycin analog. The method involves providing a cell transformed with an exogenous nucleic acid comprising a bleomycin gene cluster encoding polypeptides sufficient to direct the assembly of said bleomycin or bleomycin analog; culturing the cell under conditions permitting the biosynthesis of bleomycin or bleomycin analog; and isolating said bleomycin or bleomycin analog from said cell.

This invention also provides an isolated nucleic acid comprising a nucleic acid encoding a phosphopantetheinyl transferase said nucleic acid encoding a phosphopantetheinyl transferase being selected from the group consisting of: a nucleic acid encoding the protein encoded by the nucleic acid of SEQ ID NO:3; a nucleic acid amplified by polymerase chain reaction (PCR) using primers that specifically amplify ORF 41 (primers: SEQ ID NO:71 and SEQ ID NO:72) and *Streptomyces* nucleic acid as a template; a nucleic acid encoding a polypeptide having phosphopantetheinyl transferase activity where said nucleic acid specifically hybridizes to the nucleic acid of SEQ ID NO: 3 under stringent conditions. In one embodiment, the nucleic acid comprises the nucleic acid of SEQ ID NO:3.

In another embodiment, this invention provides a polypeptide comprising a phosphopantetheinyl transferase encoded by SEQ ID NO:3 or a polypeptide having phosphopantetheinyl transferase activity and the sequence encoded by the nucleic acid of SEQ ID NO: 3 or conservative substitutions of that polypeptide.

Also provided are vectors comprising a nucleic acid encoding a phosphopantetheinyl transferase (*e.g.*, as described above) and cells transfected with the vector.

This invention also provides a method of converting an apo carrier protein to a holo carrier protein, said method comprising reacting said apo-carrier protein with a recombinant phosphopantetheinyl transferase encoded by SEQ ID NO:3 and coenzyme A thereby producing a holo-carrier protein.

In certain embodiments, this invention specifically excludes one or more of open reading frames 1 through 41. In particularly preferred embodiments, this invention excludes open reading frames 1 through 7 (Orf 1- Orf 7).

DEFINITIONS

The "polyketide synthases" (PKSs) refers are multifunctional enzymes, related to fatty acid synthases (FASs). PKSs catalyze the biosynthesis of polyketides through repeated (decarboxylative) Claisen condensations between acylthioesters, usually acetyl, propionyl, malonyl or methylmalonyl. Following each condensation, they typically introduce structural variability into the product by catalyzing all, part, or none of a reductive cycle comprising a ketoreduction, dehydration, and enoylreduction on the β -keto group of the growing polyketide chain. PKSs incorporate enormous structural diversity into their products, in addition to varying the condensation cycle, by controlling the overall chain length, choice of primer and extender units and, particularly in the case of aromatic polyketides, regiospecific cyclizations of the nascent polyketide chain. After the carbon chain has grown to a length characteristic of each specific product, it is typically released from the synthase by thiolysis or acyltransfer. Thus, PKSs consist of families of enzymes which work together to produce a given polyketide. Two general classes of PKSs exist. One class, known as Type I PKSs, is represented by the PKSs for macrolides such as erythromycin. These "complex" or "modular" PKSs include assemblies of several large multifunctional proteins carrying, between them, a set of separate active sites for each step of carbon chain assembly and modification (Cortes *et al.* (1990) *Nature* 348: 176; Donadio *et al.* (1991) *Science* 252: 675; MacNeil *et al.* (1992) *Gene* 115: 119). Structural diversity

occurs in this class from variations in the number and type of active sites in the PKSs. This class of PKSs displays a one-to-one correlation between the number and clustering of active sites in the primary sequence of the PKS and the structure of the polyketide backbone. The second class of PKSs, called Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs typically have a single set of iteratively used active sites (Bibb *et al.* (1989) *EMBO J.* 8: 2727; Sherman *et al.* (1989) *EMBO J.* 8: 2717; Fernandez-Moreno, *et al.* (1992) *J. Biol. Chem.* 267:19278).

A "nonribosomal peptide synthase" (NRPS) refers to an enzymatic complex of eucaryotic or procaryotic origin, that is responsible for the synthesis of peptides by a nonribosomal mechanism, often known as thiotemplate synthesis (Kleinkauf and von Doehren (1987) *Ann. Rev. Microbiol.*, 41: 259-289). Such peptides, which can be up to 20 or more amino acids in length, can have a linear, cyclic (cyclosporine, tyrocidine, mycobacilline, surfactin and others) or branched cyclic structure (polymyxin, bacitracin and others) and often contain amino acids not present in proteins or modified amino acids through methylation or epimerization.

A "module" refers to a set of distinctive polypeptide domains that encode all the enzyme activities necessary for one cycle of polyketide or peptide chain elongation and associated modifications.

The terms "isolated" "purified" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. With respect to nucleic acids and/or polypeptides the term can refer to nucleic acids or polypeptides that are no longer flanked by the sequences typically flanking them in nature.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The term also includes variants on the traditional peptide linkage joining the amino acids making up the polypeptide.

The terms "nucleic acid" or "oligonucleotide" or grammatical equivalents herein refer to at least two nucleotides covalently linked together. A nucleic acid of the present invention is preferably single-stranded or double stranded and will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate

(Beaucage *et al.* (1993) *Tetrahedron* 49(10):1925) and references therein; Letsinger (1970) *J. Org. Chem.* 35:3800; Sprinzl *et al.* (1977) *Eur. J. Biochem.* 81: 579; Letsinger *et al.* (1986) *Nucl. Acids Res.* 14: 3487; Sawai *et al.* (1984) *Chem. Lett.* 805, Letsinger *et al.* (1988) *J. Am. Chem. Soc.* 110: 4470; and Pauwels *et al.* (1986) *Chemica Scripta* 26: 1419),
5 phosphorothioate (Mag *et al.* (1991) *Nucleic Acids Res.* 19:1437; and U.S. Patent No. 5,644,048), phosphorodithioate (Briu *et al.* (1989) *J. Am. Chem. Soc.* 111 :2321, O-methylphosphoroamidite linkages (*see* Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (*see* Egholm (1992) *J. Am. Chem. Soc.* 114:1895; Meier *et al.* (1992) *Chem. Int. Ed. Engl.* 31: 1008; Nielsen (1993) *Nature*, 365: 566; Carlsson *et al.* (1996) *Nature* 380: 207).
10 Other analog nucleic acids include those with positive backbones (Denpcy *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92: 6097; non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Angew. (1991) *Chem. Intl. Ed. English* 30: 423; Letsinger *et al.* (1988) *J. Am. Chem. Soc.* 110:4470; Letsinger *et al.* (1994) *Nucleoside & Nucleotide* 13:1597; Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate
15 Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker *et al.* (1994), *Bioorganic & Medicinal Chem. Lett.* 4: 395; Jeffs *et al.* (1994) *J. Biomolecular NMR* 34:17; *Tetrahedron Lett.* 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC
20 Symposium Series 580, *Carbohydrate Modifications in Antisense Research*, Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (*see* Jenkins *et al.* (1995), *Chem. Soc. Rev.* pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. These modifications of the ribose-phosphate backbone may be done to facilitate the
25 addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments.

The term "heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally associated with a region of a recombinant construct, and/or are not normally associated with a particular cell.

30 Thus, a "heterologous" region of a nucleic acid construct is an identifiable segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a

construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a host cell transformed with a construct which is not normally present in the host cell would be considered heterologous for purposes of this invention.

5 A "coding sequence" or a sequence which "encodes" a particular polypeptide (e.g. a PKS, an NRPS, *etc.*), is a nucleic acid sequence which is ultimately transcribed and/or translated into that polypeptide *in vitro* and/or *in vivo* when placed under the control of appropriate regulatory sequences. In certain embodiments, the boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop
10 codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eucaryotic mRNA, genomic DNA sequences from procaryotic or eucaryotic DNA, and even synthetic DNA sequences. In preferred embodiments, a transcription termination sequence will usually be located 3' to the coding sequence.

Expression "control sequences" refers collectively to promoter sequences,
15 ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the desired gene is capable of being transcribed and translated.

20 "Recombination" refers to the reassortment of sections of DNA or RNA sequences between two DNA or RNA molecules. "Homologous recombination" occurs between two DNA molecules which hybridize by virtue of homologous or complementary nucleotide sequences present in each DNA molecule.

The terms "stringent conditions" or "hybridization under stringent conditions"
25 refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide
30 to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes part I chapter 2 Overview of principles of hybridization and the strategy of nucleic acid probe assays*, Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence

at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see, Sambrook et al. (1989) Molecular Cloning - A Laboratory Manual (2nd ed.)* Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

A "library" or "combinatorial library" of polyketides and/or polypeptides is intended to mean a collection of polyketides and/or polypeptides (or other molecules) catalytically produced by a PKS and/or NRPS and/or hybrid PKS/NRPS (or other possible combination of synthetic elements) gene cluster. The library can be produced by a gene cluster that contains any combination of native, homolog or mutant genes from aromatic, modular or fungal PKSs and/or NRPSs. The combination of genes can be derived from a single PKS and/or NRPS gene cluster, *e.g.*, *act*, *fren*, *gra*, *tcm*, *whiE*, *gris*, *ery*, or the like, and may optionally include genes encoding tailoring enzymes which are capable of catalyzing the further modification of a polypeptide, polyketide, or other molecule.

Alternatively, the combination of genes can be rationally or stochastically derived from an assortment of NRPS and/or PKS gene clusters. The library of polyketides and/or polypeptides and/or other molecules thus produced can be tested or screened for biological, pharmacological or other activity.

By "random assortment" is intended any combination and/or order of genes, homologs or mutants which encode for the various PKS and/or NRPS enzymes, modules, active sites or portions thereof derived from aromatic, modular or fungal PKS and/or NRPS gene clusters.

5 By "genetically engineered host cell" is meant a host cell where the native PKS and/or NRPS gene cluster has been altered or deleted using recombinant DNA techniques or a host cell into which a heterologous PKS and/or NRPS and/or hybrid PKS/NRPS gene cluster has been inserted. Thus, the term would not encompass mutational events occurring in nature. A "host cell" is a cell derived from a procaryotic microorganism
10 or a eucaryotic cell line cultured as a unicellular entity, which can be, or has been, used as a recipient for recombinant vectors bearing the PKS, NRPS, and/or hybrid gene clusters of the invention. The term includes the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due
15 to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired PKS, are included in the definition, and are covered by the above terms.

Expression vectors are defined herein as nucleic acid sequences that are direct
20 the transcription of cloned copies of genes/cDNAs and/or the translation of their mRNAs in an appropriate host. Such vectors can be used to express genes or cDNAs in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells. Expression vectors include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Specifically designed vectors allow the shuttling of DNA
25 between hosts, such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector preferably contains: an origin of replication for autonomous replication in a host cell, a selectable marker, optionally one or more restriction enzyme sites, optionally one or more constitutive or inducible promoters. In preferred embodiments, an expression vector is a replicable DNA construct in which a DNA sequence encoding a one or more PKS
30 and/or NRPS domains and/or modules is operably linked to suitable control sequences capable of effecting the expression of the products of these synthase and/or synthetases in a suitable host. Control sequences include a transcriptional promoter, an optional operator sequence to control transcription and sequences which control the termination of transcription and translation, and so forth.

A "bleomycin open reading frame", or "bleomycin ORF", or "*BLM* Orf" refers to a nucleic acid open reading frame that encodes a polypeptide or polypeptide domain that has an enzymatic activity used in the biosynthesis of a bleomycin.

A "PKS/NRPS/PKS" system refers to a synthetic system comprising an NRPS
5 flanked by two PKSs. A "NRPS/PKS/NRPS" system refers to a synthetic system comprising a PKS flanked by two NRPSs. A "hybrid PKS/NRPS system" or a "hybrid NRPS/PKS system" refers to a hybrid synthetic system comprising at least one PKS and one NRPS module. The system can comprise multiple modules and the order can vary.

A "biological molecule that is a substrate for a polypeptide encoded by a
10 bleomycin biosynthesis gene" refers to a molecule that is chemically modified by one or more polypeptides encoded by open reading frame(s) of the *blm* gene cluster. The "substrate" may be a native molecule that typically participates in the biosynthesis of a bleomycin, or can be any other molecule that can be similarly acted upon by the polypeptide.

A "polymorphism" is a variation in the DNA sequence of some members of a
15 species. A polymorphism is thus said to be "allelic," in that, due to the existence of the polymorphism, some members of a species may have the unmutated sequence (*i.e.* the original "allele") whereas other members may have a mutated sequence (*i.e.* the variant or mutant "allele"). In the simplest case, only one mutated sequence may exist, and the polymorphism is said to be diallelic. In the case of diallelic diploid organisms, three
20 genotypes are possible. They can be homozygous for one allele, homozygous for the other allele or heterozygous. In the case of diallelic haploid organisms, they can have one allele or the other, thus only two genotypes are possible. The occurrence of alternative mutations can give rise to triallelic, *etc.* polymorphisms. An allele may be referred to by the nucleotide(s) that comprise the mutation.

25 "Single nucleotide polymorphism" or "SNPs are defined by their characteristic attributes. A central attribute of such a polymorphism is that it contains a polymorphic site, "X," most preferably occupied by a single nucleotide, which is the site of the polymorphism's variation (Goelet and Knapp U.S. patent application Ser. No. 08/145,145). Methods of identifying SNPs are well known to those of skill in the art (*see*,
30 *e.g.*, U.S. Patent 5,952,174).

The following abbreviations are used herein:: A, adenylation; ACP, acyl carrier protein; AT, acyltransferase; BLM, bleomycin; C, condensation; Cy, condensation/cyclization; KR, ketoreductase; KS, ketoacyl synthase; MT, methyltransferase; NRPS, nonribosomal peptide synthetase; orf, open reading frame; Ox, oxidation; PCP,

peptidyl carrier protein; PCR, polymerase chain reaction; PKS, polyketide synthase; *Sv*, *Streptomyces verticillus*, ArCP, aryl carrier protein, bp, base pair, CoA, co-enzyme A, DTT, dithiothreitol; FAS, fatty acid synthase; kb, kilobase; PPTase, 4'-phosphopantetheinyl transferase; TCA, trichloroacetic acid; and DEBS, 6-deoxyerythronolide B synthase..

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BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate the biosynthetic pathway for bleomycin in *Sv* (ATCC 15003). Figure 1A illustrates a biosynthetic pathway for BLM in *Sv* ATCC15003—intermediates except those in brackets were identified. Figure 1B shows a linear model for the Blm megasynthetase-templated assembly of the BLM peptide/polyketide/peptide aglycone from nine amino acids and one acetate—shaded circles represent atypical domains carrying out the proposed novel chemistry, and arrows with broken line indicate where biosynthetic intermediates were derailed. Three-letter amino acid designations were used. [HO], hydroxylation; [H], reduction.

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Figure 2 provides a restriction map and gene organization of the *blm* gene cluster from *Sv* ATCC15003 (B, *Bam*HI). Proposed functions for individual open reading frames are summarized in Tables I and II. Modules for individual NRPS and PKS were given along with their proposed substrates in parentheses.

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Figures 3A, 3B, 3C, and 3D illustrate the determination of substrate specificity for NRPS-1 and NRPS-6. Figure 3A shows a comparison of the A3 to A6 region of A domains to 84 NRPS modules available at GenBank that activate various amino acids. Figure 3B shows a comparison of amino acid residues that putatively line the substrate binding pockets for A domains (single-letter amino acid designations were used). The number following the protein name indicates the order of a particular A domain in the multimodular NRPS protein. The protein accession numbers are P48663 (HMWP2), P19828 (AngR), AAC06346 (BacA-2), CAB03756 (MbtB), 3510629 (SyrE-7), 3114612 (AcmB-1), CAA67248 (SnbC-1), and 3560507 (FxbC-2). Dhb stands for 2,3-dehydroaminobutyric acid. It is not known if Dhb is the direct substrate for SyrE-7 or resulted from dehydration of an SyrE-7 activated Thr (Guenzi *et al.* (1998) *J. Biol. Chem.* 273: 32857-32863). Figure 3C illustrates purified proteins after overexpression in *E. coli* as analyzed by electrophoresis on a 10% SDS-polyacrylamide gel (the calculated molecular weights for NRPS-1A and NRPS-6A are 64,212 and 61,899, respectively). Figure 3D illustrates substrate specificities as determined by the ATP-PPi exchange reaction with the amino acids of BLM as substrates

(100% relative activity corresponds to 103,000 cpm for NRPS-1A and 256,000 cpm for NRPS-6A).

Figure 4 illustrates a three-module NRPS/PKS/NRPS model for channeling the growing intermediate between NRPS and PKS modules and between PKS and NRPS modules. The KS, ACP, and C domains are shaded to emphasize their unique activities that are responsible for elongating a growing peptide with a short carboxylic acid and a growing polyketide with an amino acid in hybrid peptide/polyketide/peptide biosynthesis.

Figure 5 illustrates the use of *blmVIII* methyltransferase domain to introduce branched methyl groups in a polyketide synthesis. PCK12 has been described by Kao *et al.* (1995) *J. Am. Chem. Soc.*, 7: 9105-9106. DE-1, DE-2 and DE-3 are three representative products demonstrating the strategy and utility of *blmVIII* in introducing a CH₃ group in polyketide biosynthesis.

Figure 6 illustrates the use of the *blm* NRPS and PKS enzymes to synthesize a variety of hybrid polyketide/peptide molecules including, but not limited to, a family of oxazolines/oxazoles, and thiazoline/thiazoles.

Figure 7 illustrates the use of elements of the *blm* gene cluster to synthesize various sugars.

Figure 8A shows a restriction map of the *blm* gene cluster from *Sv* ATCC15003 (B, *Bam*HI). 8B shows the relative position of the *blmI*, *blmII*, and *blmXI* genes to the two *blmAB* resistance genes (*blm^R*, Blm resistance). Individual open reading frames are represented by open arrows. Figure 8C shows the nucleotide sequence of the *blmI* gene. The potential ribosome-binding site (RBS) and the conserved motif for 4'-phosphopantetheinylation are underlined. The sequence has been deposited into GenBank under accession no. _____.

Figure 9 shows an amino acid sequence comparison of BlmI with PCP domains of known type I NRPSs (Grs-2 [P14688], 36% identity, 58% similarity; Srfa-3 [Q08787], 40% identity, 64% similarity; Vir-s [Y11547], 36% identity, 60% similarity; Saf-b [U24657], 40% identity, 54% similarity). Given in brackets are nucleotide sequence accession numbers. The shaded letters indicate similar amino acids. Consensus residues are amino acids that are similar in more than three sequences. The signature motif for 4'-phosphopantetheinylation is underlined.

Figures 10A and 10B show the HPLC analysis of BlmI purified from *E. coli* OG7001(pBS2) (Fig. 10A), and *E. coli* OG7001(pBS2/pDPT-Gsp) (Fig. 10B).

Figure 11 shows the enzyme architecture of type I and type II PKS and NRPS. A, adenylation domain; ACP, acyl carrier protein or ACP domain; AT, acyl transferase; C, condensation protein or C domain; KS, β -ketoacyl synthase domain; KS α , β -ketoacyl synthase α subunit; KS β , β -ketoacyl synthase β subunit; PCP, peptidyl carrier protein or PCP domain.

Figure 12 illustrates the reaction catalyzed by phosphopantetheinyl transferases (PPTases).

Figure 13 shows a restriction map and gene organization of the *pptA* locus from *Sv* ATCC15003

DETAILED DESCRIPTION

Polyketides and polypeptides can be assembled in a remarkably similar manner by repetitive addition of an extending unit to a growing chain by polyketide synthases (PKS) and nonribosomal peptide synthetase (NRPS) respectively. In the case of polyketides, the extending unit is typically a fatty acid (activated as an acyl CoA thioester) while the extending unit for polypeptides is typically an amino acid (activated as an aminonacyl adenylate). Both the PKS and NRPS systems have evolved a modular organization to define the number, sequence, and specificity of the incorporation of the extending unit and utilized the 4'-phosphopantetheine prosthetic group to channel the growing intermediate during the elongation process.

This invention pertains to the discovery that a PKS-bound growing polyketide intermediate could be further elongated by an NRPS module, or conversely, a NRPS-bound growing polypeptide intermediate can be further elongated by a PKS module. This discovery permits the exploitation of NRPS, PKS, and hybrid NRPS/PKS systems to provide a number of novel hybrid peptide/polyketide metabolites from amino acids and short fatty acids.

It was also a discovery of this invention that this hybrid NRPS/PKS/NRPS system is exemplified by the bleomycin (Blm) biosynthesis pathway in *Streptomyces verticillus* (*Sv*) (ATCC 15003). The bleomycins are a family of glycopeptide-derived antibiotics originally isolated by Umezawa in 1996 from the fermentation broth of *S. verticillus*. Bleomycins (BLMs) exhibit strong anti-tumor activity are currently used in the treatment of lymphoma, particularly Hodgkin's disease, testicular tumors, squamous cell carcinomas of skin, head, cervix, penis, rectum, and for intracavitary therapy of malignant effusions in ovarian and breast cancer. The commercial product, Blenoxane®, contains

BLM A2 and B2 as the principle constituents. Almost uniquely among anticancer drugs, BLM does not cause myelosuppression, promoting its wide application in combination chemotherapy.

In one aspect, this invention provides a cloned and characterized BLM gene cluster consisting of characteristic NRPS and PKS genes from the Blm producer *Streptoveticillum sp.* (ATCC 15003). The cloned and isolated Blm gene cluster provides a method of recombinantly expressing bleomycin and/or bleomycin analogues. Thus, in one embodiment, this invention provides for nucleic acids encoding bleomycin synthetic machinery or subunits thereof, for cells recombinantly modified to express a bleomycin and/or bleomycin analogue, and for a bleomycin or bleomycin analogue recombinantly expressed in such cells.

Like other polyketide synthase or nonribosomal peptide synthetases, the bleomycin synthetic pathway is organized into modules, each module catalyzing the addition and/or modification of one subunit (*e.g.* fatty acid or amino acid). Each module is organized into a number of domains each domain having a characteristic activity (*e.g.* activation, condensation, condensation/cyclization, *etc.*). The catalytic domains within a module and the modules themselves are often arranged collinearly and the order of biosynthetic modules from NH₂- to COOH-terminus on each PKS and NRPS polypeptide and the number and type of catalytic domains within each determine the order of structural and functional elements in the resulting product. The size and complexity of the ultimately formed product are controlled by the number of repeated acyl chain extension steps that are, in turn, a function of the number and placement of carrier protein domains in these multimodular enzymes. The number composition and order of such domains can be altered either to introduce modifications, *e.g.* into the bleomycin to produce bleomycin analogues, or to produce different or completely new molecules. Such "recombination" is not restricted solely to recombination among the bleomycin catalytic domains and/or modules, but can also involve recombination between bleomycin modules and/or subunits and other PKS and/or NRPS modules and/or subunit. Moreover the discovery that synthetic pathways can incorporate both PKS and NRPS modules and/or catalytic domains makes available hybrid PKS/NRPS syntheses.

Thus, in one embodiment this invention contemplates the use of *blm* gene cluster modules and/or catalytic domains to make various peptide and/or polyketide, and/or hybrid polypeptide/polyketide metabolites (including, but not limited to bleomycin

intermediates or shunt metabolites), in combinatorial biosynthesis with other polyketide synthases and/or other nonribosomal peptide synthetases.

The *blm* gene cluster contains several glycosylases which can be used alone or in context with other PKS and/or NRPS modules or catalytic domains to make various metabolites with sugars associated with bleomycins (bleomycin sugars).

In addition, the *blm* gene cluster includes a novel methyltransferase domain that can be used to make polyketide metabolites with methyl branch(s).

The *blm* gene cluster also is characterized by the unusual Cy domains as well as the unprecedented Ox domain (see, e.g. BlmIV and BlmIII NRPSs), providing an efficient biosynthesis for a bithiazole structure. The *blm* gene cluster, *blm* modules, or *blm* catalytic domains can be used either individually or collectively (alone or in combinations with other nonribosomal peptide synthetases or polyketide synthases) to make thiazolidine, thiazoline and thiazole, bi-thiazolidine, bithiazoline, and bithiazole-containing microbial metabolites.

Other uses include, but are not limited to the usage of the *blm* gene cluster/modules/catalytic units (either individually or collectively) or the Blm model to make heterocyclic ring-containing microbial metabolites, such as five member S- and N-containing compounds of the thiazolidine, thiazoline and thiazole family or the O- and N-containing compounds of the oxazolidine, oxazoline, and oxazole family or to make sugars, such L-sugars (with the BlmG epimerase), sugars modified by carbamoyl group (with BlmD), and disaccharides.

This invention also includes the discovery of a novel discrete PCP protein (encoded by the *BlmI* gene). Apo-BlmI can be efficiently modified into holo-BlmI either *in vivo* or *in vitro* by PCP-specific 4'-phosphopantetheine transferases (PPTases) such as Gsp and Sfp. Unlike the PCP domains in type I NRPSs, blmI lacks its cognate A domain and can be aminoacylated by Val-A, an A domain from a completely unrelated type I NRPS. BlmI, therefore, represents the first characterized type II PCP, providing the genetic and biochemical evidence to support the existence of a type II NRPS. The latter system is useful, in a manner analogous to the type I NRPS, *i.e.*, modular NRPS, in the combinatorial manipulation of NRPS proteins to generate novel peptides. This invention also includes the discovery and characterization of a novel PPTase (encoded by the *pptA* gene in Figure 13). This PPTase can be used in engineered biosynthesis of polyketides, peptides, hybrid peptide and polyketide metabolites, hybrid polyketide and peptide metabolites, or the combination of both types of metabolites. The PPTase can also be used in converting apo-peptidyl carrier

proteins (both type I and type II) and acyl carrier proteins (both type I and type II) into the holo-proteins.

The Examples provided herein and the accompanying primers permit one of ordinary skill in the art to isolate the *blm* gene cluster of this invention, its constituent ORFs, various modules, or enzymatic domains. The isolated nucleic acid components can be used to express one or more polypeptide components for *in vivo* (e.g. recombinant) synthesis of one or more polypeptides and/or polyketides as indicated above. It will also be appreciated that the *blm* cluster polypeptides can be used for *ex vivo* assembly of various macromolecules.

I. BLM gene cluster and the PPTase gene.

A) The BLM gene cluster.

The nucleic acids comprising the *blm* gene cluster are identified in Tables I and II and listed in the sequence listing provided herein (SEQ ID NOS: 1 and 2, GenBank Accession numbers AT-149091, AT-210249, AF210311). In particular, Table I identifies genes and functions of open reading frames (ORFs) responsible for the biosynthesis of the hybrid peptide/polyketide/peptide backbone and sugar moieties of bleomycin, while Table II identifies a number of ORFs comprising the *blm* gene cluster, identifies the activity of the catalytic domain encoded by the ORF and provides primers for the amplification and isolation of that orf.

As illustrated in Example 1, the *blm* cluster comprises a PKS module, flanked by several NRPS modules along with several sugar biosynthesis genes and genes encoding other biosynthesis enzymes as well as several resistance and regulatory genes (Table 1).

Table I. Determined functions of ORFs in the bleomycin biosynthesis gene cluster

Gene	Amino acids	Sequence Homolog ¹	Proposed function ^{2,3}
<i>orf8</i>	424	YqeR (BAA12461)	Oxidase
<i>blmC</i>	498	RfaE (AA07904.1)	NDP-glucose synthase
<i>blmI</i>	90	GrsB (P14688)	Type II PCP
<i>blmD</i>	545	NodU (Q53515)	Carbamoyl transferase
<i>blmE</i>	390	RfaF (AAD16056)	Glycosyl transferase
<i>orf13</i>	187	MbtH (O05821)	Unknown
<i>blmII</i>	462	Nrp (CAA98937)	NRPS condensation enzyme
<i>orf15</i>	339	SyrP (1890776)	Regulation
<i>blmII</i>	935	HMWP2 (P48633), McbC (P23185)	A PCP Ox

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<i>blmIV</i>	2626	HMWP2 (P48633)	C A PCP Cy A PCP Cy
<i>orf18</i>	638	AsnB (2293165)	Asparagine synthetase
<i>blmF</i>	494	RfbC (Q50864)/BlmOrf1 (507319)	Glycosyl transferase/ β -hydroxylase
<i>blmG</i>	325	YtdB (2293288)	Sugar epimerase
<i>blmV</i>	645	McyB (2708278)	PCP C
<i>blmVI</i>	2675	ACoAS (1658531), PksD (S73014) SnbDE (CAA67249)	A ⁴ ACP C A PCP C A
<i>blmVII</i>	1218	SyrE (3510629)	C A PCP
<i>blmVIII</i>	1841	HMWP1 (CAA73127)	KS AT MT KR ACP
<i>blmIX</i>	1066	SafB (1171128)	C A PCP
<i>blmX</i>	2140	TycC (2623773)	C A PCP C A PCP
<i>blmXI</i>	688	SyrE (3510629)	NRPS condensation enzyme
<i>orf28</i>	239	SC9C7.04C (CAA22716)	Unknown
<i>orf29</i>	582	YvdB (CA08068)	Transmembrane transporter
<i>orf30</i>	113	SmtB (P30340)	Regulation
<i>orf31</i>	117	PhnA (P16680)	Unknown

1. Protein accession numbers are given in parentheses. 2. Underlined domains contain motifs that are clearly different from known NRPS or PKS domains. 3. This A domain lacks the typical NRPS A1, A2, A4, A8, and A9 motifs and more closely resembles acyl CoA synthases. *ORF1* to *ORF7* were reported by Schmidt (1994) *Gene* 151:17-21, who assigned *ORF2* as *blmA* and *ORF4* as *blmB*.

Noteworthy are the genes encoding the NRPS and PKS enzymes. The *blmI*, *blmII*, and *blmXI* genes encode NRPSs with an unusual architecture. In contrast to all known NRPSs, which are of modular organization with each module consisting minimally of a condensation (C), an adenylation (A), and a peptidyl carrier protein (PCP) domain, *BlmI*, *BlmII*, and *BlmXI* are discrete proteins homologous to individual domains of type I NRPSs. We have characterized *BlmI* as a type II PCP (Du and Shen (1999) *Chem. Biol.* 6: 507-517). The *BlmII* and *BlmXI* proteins can serve as candidates for type II condensation enzymes.

The *blmIII*, *blmIV*, *blmV*, *blmVI*, *blmVII*, *blmIX*, and *blmX* genes encode modular NRPSs consisting of domains characteristic for known type I NRPSs, such as the A, PCP, C, and condensation/cyclization (Cy) domains, as well as an unprecedented oxidation (Ox) domain. *BlmVI* is unique among all the *Blm* NRPSs identified. Its N-terminal module (NRPS-5) consists of an atypical A domain, which bears a close resemblance to a family of acyl CoA synthases (Fitzmaurice and Kolattukudy (1997) *J. Bacteriol.* 179: 2608-2615; Fitzmaurice and Kolattukudy (1998) *J. Biol. Chem.* 273: 8033-8039), and an acyl carrier protein (ACP)-like domain. Its C-terminal module is truncated and presumably interacts with *BlmV* to constitute the complete NRPS-3 module (Fig. 1B). Also noteworthy are the C domain of NRPS-3 that lacks both His residues of the conserved HHxxxDG (SEQ ID NO: 4) active site for transpeptidation (Stachelhaus *et al.* (1998) *J. Biol. Chem.* 273: 22773-22781)

and the extra C domain at the C-terminus of BlmV. These unusual features associated with BlmVI and BlmV may play roles in the formation of the β -aminoalaninamide and the pyrimidine moieties of BLM, which are unprecedented in peptide biosynthesis.

The *blmVIII* gene encodes a PKS module consisting of domains characteristic for known PKSs, such as ketoacyl synthase (KS), acyltransferase (AT), ketoreductase (KR), and ACP, with malonyl CoA acting as an extending unit according to sequence comparison of the AT domain (Haydock *et al.* (1995) *FEBS Lett.* 374: 246-248) (Fig. 1B).

The identification of an integrated methyltransferase (MT) domain in the middle of *BlmVIII* is unique, representing the first PKS from actinomycetes that contains an internal MT domain.

Table II. *Blm* gene cluster open reading frames (ORFs) and primers for ORF amplification.

Orf #	Position	Activity	Method	Primers Forward Reverse	Se q ID No
orf-8	76183-77457	Oxygen-independent coproporphyrinogen III oxidase	Gapped-blast comparison ¹	F: ATGAGCCACGCCATCGGA R: TCAGGCGCGTTCGGGGGC	5 6
orf-9	74690-76186	ADP-heptose synthase (<i>blmC</i>)	Gapped-blast comparison ¹	F: GTGAACACCGACCTGCCC R: TCATGGGGTGTCTCCCTC	7 8
orf-10	74421-74693	Peptidyl carrier protein (<i>blmI</i>)	Expression and biochemical characterization. ²	F: ATGAGCGCCCCGCGGGGC R: TCACCGGTCCCCGCTCCCC	9 10
orf-11	72787-74424	Carbamyltransferase (<i>blmD</i>)	Gapped-blast comparison ¹	F: ATGAGCGCCGACCCGTCC R: TCATGAGCGGGCCGCCGT	11 12
orf-12	71618-72790	ADP-heptose:LPS heptosyl transferase (<i>blmE</i>)	Gapped-blast comparison ¹	F: ATGACCACCCCATGACC R: TCATGGGGTACTCCTGAT	13 14
orf-13	70983-71546	Homolog of mbtH in the synthesis of mycobactin	Gapped-blast comparison ¹	F: ATGACCACGACCCCGCGG R: TCAGGTGCCGGACACGCG	15 16
orf-14	69598-70986	Peptide synthetase (condensation, <i>blmII</i>)	Gapped-blast comparison ¹	F: GTGACCGCCCCCGGCACA R: TCATCGGTGGCTCCTCGT	17 18
orf-15	68582-69601	Regulatory gene (homolog of <i>syrP</i>)	Gapped-blast comparison ¹	F: GTGAACCGGCACGGCCCC R: TCACGCGCTCACCTCGTC	19 20
orf-16	65778-68585	Mutated peptide synthetase-oxidase (NRPS-0, <i>blmIII</i>)	Gapped-blast comparison ¹	F: GTGACGAGCGCCCGGCC R: TCACGGGGCCTCCGTGCG	21 22
orf-17	57901-65781	Peptide synthetase (NRPS-2-1, <i>blmIV</i>)	Expression and biochemical characterization. ²	F: ATGCTGCACGGCGCCGCG R: TCACTCCGGTCCACCTCC	23 24

orf-18	55899-57815	Asparagine synthetase	Gapped-blast comparison ¹	F: GTGAGGCCCGTGTGCGGC R: TCAGCCACCGTTGCCCGC	25 26
orf-19	54418-55902	Homolog of hydroxylase-dehydrogenase (<i>blmF</i>)	Gapped-blast comparison ¹	F: GTGAAGGACCTCGGCCGG R: TCACTCCCCCGGTGCCCG	27 28
orf-20	53427-54404	Nucleotide-sugar epimerase (<i>blmG</i>)	Gapped-blast comparison ¹	F: GTGACATGGACCGTGGTG R: TCAGGCATCGGCCCTCCC	29 30
orf-21	51493-53430	Peptide synthetase (NRPS-3CT, <i>blmV</i>)	Gapped-blast comparison ¹	F: ATGCGCGGGCATGACGAC R: TCACGGTGTCTCTCCCTC	31 32
orf-22	43263-51290	Peptide synthetase (NRPS-5-4-3, <i>blmVI</i>)	Expression and biochemical characterization. ²	F: ATGAGCCGGCCGGCCGGC R: TCATGCTCGGTCATCGCC	33 34
orf-23	39610-43266	Peptide synthetase (NRPS-6, <i>blmVII</i>)	Expression and biochemical characterization. ²	F: GTGACCACGCCCCGCATC R: TCATTGCGGACGCGGGCA	35 36
orf-24	34088-39613	Polyketide synthase (<i>blmVIII</i>)	Gapped-blast comparison ¹	F: ATGAGCCATGCCGACGCG R: TCACAGCACCACTCTTC	37 38
orf-25	30891-34091	Peptide synthetase (NRPS-7, <i>blmIX</i>)	Gapped-blast comparison ¹	F: ATGACCCCGGCCGCGAC R: TCATCGTCCGCCGCTTT	39 40
orf-26	24406-30894	Peptide synthetase (NRPS-9-8, <i>blmX</i>)	Gapped-blast comparison ¹	F: ATGCCTCGGTGTGCCCGA R: TCATTGCGCGGCACCTCC	41 42
orf-27	22127-24193	Peptide synthetase (condensation, <i>blmXI</i>)	Gapped-blast comparison ¹	F: GTGGGTTTCCGTCGAGCG R: TTACACCCTCCGTTTCTC	43 44
orf-28	21367-22086	Phosphatidylserine decarboxylase	Gapped-blast comparison ¹	F: ATGGCACAGGACCTGAAC R: TCAACGCCACCGGATCTT	45 46
orf-29	19161-20909	Transmembrane transporter	Gapped-blast comparison ¹	F: GTGAGCTCCCTCGCCGTC R: TCATCGTCGGGCACTCGG	47 48
orf-30	18823-19164	Metal dependent regulatory element	Gapped-blast comparison ¹	F: GTGCCGGTTCCGCTGTAT R: TCACCGGGCACTGACCTC	49 50
orf-31	18660-18307	PHNA homolog	Gapped-blast comparison ¹	F: GTGACCGAGAACCTTCCG R: TCAGACCTTCTTGACCAC	51 52
orf-32	17736-9211	Peptide synthetase (NRPS-11-10)	Gapped-blast comparison ¹	F: ATGGCCTCAGACGCTTTG R: TCATTGAGACTCCTCCTC	53 54
orf-33	9214-7859	Putative transporter	Gapped-blast comparison ¹	F: ATGATGAAGTCAAGCCGC R: TCAGTGGCTTACAAGGAG	55 56
orf-34	7797-6784	Homolog of clavaminic acid synthase	Gapped-blast comparison ¹	F: ATGACTGACCTGCCGTTG R: TCACACCAGCAGCGAGGT	57 58
orf-35	6773-6021	Thioesterase	Gapped-blast comparison ¹	F: ATGGATTTCCTCCCTCACC R: TCATGCCCTACCTCGGC	59 60
orf-36	6024-4741	Putative transporter	Gapped-blast comparison ¹	F: ATGACCGCGCGCTCGAC R: TCACTCCTCGGCTTCGGC	61 62
orf-37	4733-3915	Unknown	Gapped-blast comparison ¹	F: GTGTCCAAGAACGCGGCG R: TCATCGGCTCGCCTCGTG	63 64
orf-38	3918-2182	Peptide synthetase (NRPS-12)	Gapped-blast comparison ¹	F: ATGACCTCACCCTGCGG R: TCACTCGGGCACTCCTTC	65 66
orf-39	2185-1199	Regulatory gene (homolog of <i>SyrP</i>)	Gapped-blast comparison ¹	F: GTGACCGGTTCCGTAACG R: TCATGAGTCCGCCGAGGT	67 68
orf-	1015-1	Peptide synthetase	Gapped-blast	F: ATGACAGAGGTCCGAGGT	69

Sub
A6
cont

See 26 cont

40			comparison ¹	R: CCCGGCAACCGCCCTCCC	70
orf-41	On a separate sequence	4'-phosphopantetheinyl transferase (<i>pptA</i>)	Expression and biochemical characterization. ²	F: GTGATCGCCGCCCTCCTG R: TTACGGGACGGCGGTCCG	71 72

The Blm megasynthetase comprises nine NRPS modules and one PKS module forming a hybrid NRPS/PKS/NRPS metasyntetase (Fig. 1A). Inspection of the blm gene cluster (Fig. 2) showed that the Blm NRPS and PKS modules apparently are not organized according to the "colinearity rule" for BLM biosynthesis (Fig. 1). Detailed functional organization of the megasynthetase and the BLM synthetic pathway is provided in Example I.

B) PPTase

This invention also provides the gene (*pptA*, Fig. 13) encoding phosphopantetheine transferase (PPTase) (GenBank Accession No: AF210311) (*see*, SEQ ID NO: 3). PPTase converts carrier proteins for the growing acyl chain from inactive apo-forms to functional holo-forms by the covalent attachment of the 4'-phosphopantetheine moiety of coenzyme A to a conserved serine residue of the carrier-protein substrate (*see, e.g.*, Fig. 1A).

Using the sequence information provided herein (*e.g.* primer sequences and PPTase sequence) the PPTase nucleic acids can be routinely isolated according to standard methods (*e.g.* PCR amplification). Detailed protocols for the isolation of the PPTase are provided in Example 3.

Other PPTases can be identified using the probes and primers illustrated in Example 3. Briefly, using a primer to the THC motif (5'-C GGC ATG GTC GGC TCC HTN CAN CAY TG -3' (SEQ ID NO: 73) where H= C+A, N = A + C + T + G, Y = C + T, K = G + T, R = A + G, W = T + A), and a primer designed around the typical C-terminal PPTase motif (*e.g.*, KEA-1: 5'-T GCA GCA GAA CAG GAG GCK NYC CCA NKG - 3' (SEQ ID NO: 74) and KEA-2: 5'- TG GGT CAG CGG GTA CCA NRC YTT RWA - 3' (SEQ ID NO: 75)), and using *S. verticillus* chromosomal DNA as template, the set of primers THC/KEA-2 a probe can be amplified (about 250 bp), that specifically binds to a PPTase. Libraries of organisms comprising NRPS, PKS, and/or hybrid PKS/NRPS pathways can be probed for the presence of a PPTase sequence. Once hybridizing clones are identified, the PPTase sequence can be isolated according to standard methods well know to those of skill in the art (*see, e.g.*, Example 3).

C) Isolation/preparation of nucleic acids.

In one embodiment, this invention provides nucleic acids for the recombinant expression of a bleomycin. Such nucleic acids include isolated gene cluster(s) comprising open reading frames encoding polypeptides sufficient to direct the assembly of a bleomycin.

5 In other embodiments of this invention, modified bleomycins (*e.g.* bleomycin analogs), novel polyketides, polypeptides, and combinations thereof (polyketide/polypeptide hybrids) are created by modifying PKSs and/or NRPSs so as to introduce variations into known polymers synthesized by the enzymes. Such variations may be introduced by design, for example to modify a known molecule in a specific way, *e.g.* by replacing a single
10 monomeric unit within a polymer with another, thereby creating a derivative molecule of predicted structure. Alternatively, variations can be made randomly, for example by making a library of molecular variants of a known polymer by systematically or haphazardly replacing one or more modules or enzymatic domains in a known PKS or NRPS with a collection of alternative modules or domains. Production of alternative/modified PKSs,
15 NRPSs and hybrid systems is described below.

Using the primer and sequence information provided herein, one of ordinary skill in the art can routinely isolate/clone the PKS and/or NRPS modules and/or enzymatic domains described herein. For example, the PCR primers provided in Table II, above, can be used to amplify any of the orfs identified therein. Moreover, using the sequence
20 information for the *blm* gene cluster provided herein, the design of other primers suitable of the amplification of individual ORFs, combinations of ORFs, genes, *etc.* is routine.

Typically such amplifications will utilize the DNA of an organism containing the requisite genes (*e.g.* *Streptomyces verticillus*) as a template. Typical amplification conditions include a PCR mixture consisting of 5 ng of *S. verticillus* genomic or plasmid
25 DNA as template, 25 pmoles of each primers, 25 μ M dNTP, 5% DMSO, 2 units of *Taq* polymerase, 1 x buffer, with or without 20% glycerol in a final volume of 50 μ L. PCR is carried out (*e.g.* on a Gene Amp PCR System 2400 (Perkin-Elmer/ABI)) with a cycling scheme as follows: initial denaturing at 94°C for 5 min, 24-36 cycles of 45 sec at 94°C, 1 min at 60°C, 2 min at 72°C, followed by additional 7 min at 72°C. One of skill will
30 appreciate that optimization of such a protocol, *e.g.* to improve yield, *etc.* is routine (*see, e.g.*, U.S. Patent No. 4,683,202; Innis (1990) *PCR Protocols A Guide to Methods and Applications* Academic Press Inc. San Diego, CA, *etc.*). In addition, primer may be designed to introduce restriction sites and so facilitate cloning of the amplified sequence into a vector.

Using the information provided herein other approaches to cloning the desired sequences will be apparent to those of skill in the art. For example, the PKS or NRPS modules or enzymatic domains of interest can be obtained from an organism that expresses the same, using recombinant methods, such as by screening cDNA or genomic libraries, derived from cells expressing the gene, or by deriving the gene from a vector known to include the same. The gene can then be isolated and combined with other desired NRPS and/or PKS modules or domains, using standard techniques. If the gene in question is already present in a suitable expression vector, it can be combined *in situ*, with, e.g., other PKS subunits, as desired. The gene of interest can also be produced synthetically, rather than cloned. The nucleotide sequence can be designed with the appropriate codons for the particular amino acid sequence desired. In general, one will select preferred codons for the intended host in which the sequence will be expressed. The complete sequence can be assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence (*see, e.g.*, Edge (1981) *Nature* 292:756; Nambair *et al.* (1984) *Science* 223: 1299; Jay *et al.* (1984) *J. Biol. Chem.* 259:6311). In addition, it is noted that custom gene synthesis is commercially available (*see, e.g.* Operon Technologies, Alameda, CA).

Examples of such techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel (1989) *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY; Ausubel (1994) *Current Protocols in Molecular Biology*, Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., U.S. Patent 5,017,478; and European Patent No. 0,246,864.

II. Expression of blm gene clusters, modules, and enzymatic domains.

As indicated above, in one embodiment this invention provides novel NRPS and PKS genes for the efficient recombinant production of both novel and known polyketides, peptides, and polyketide/polypeptide hybrids by expressing them *in vivo*. In other embodiments, such syntheses are carried out *in vitro*. Even *in vitro* syntheses, however, typically utilize recombinantly expressed PKSs, NRPSs, or enzymatic domains thereof. Thus, it is frequently desirable to express protein components of the PKSs or NRPs described above.

Typically expression of the protein components of the pathway and/or of the products of the NRPS/PKS pathway is accomplished by placing the subject PKS or NRPS nucleic acid(s) in an expression vector, and transfecting a cell with the vector such that the cell expresses the desired product(s).

5 **A) Expression vectors**

The choice of vector depends on the sequence(s) that are to be expressed. Any transducible cloning vector can be used as a cloning vector for the nucleic acid constructs of this invention. However, where large clusters are to be expressed, it phagemids, cosmids, PIs, YACs, BACs, PACs, HACs or similar cloning vectors be used for
10 cloning the nucleotide sequences into the host cell. Phagemids, cosmids, and BACs, for example, are advantageous vectors due to the ability to insert and stably propagate therein larger fragments of DNA than in M13 phage and lambda phage, respectively. Phagemids which will find use in this method generally include hybrids between plasmids and filamentous phage cloning vehicles. Cosmids which will find use in this method generally
15 include lambda phage-based vectors into which cos sites have been inserted. Recipient pool cloning vectors can be any suitable plasmid. The cloning vectors into which pools of mutants are inserted may be identical or may be constructed to harbor and express different genetic markers (see, *e.g.*, Sambrook *et al.*, *supra*). The utility of employing such vectors having different marker genes may be exploited to facilitate a determination of successful
20 transduction.

In preferred embodiments of this invention, vectors are used to introduce PKS, NRPS, or NRPS/PKS genes or gene clusters into host (*e.g. Streptomyces*) cells. Numerous vectors for use in particular host cells are well known to those of skill in the art. For example described in Malpartida and Hopwood, (1984) *Nature*, 309:462-464; Kao *et al.*,
25 (1994), *Science*, 265: 509-512; and Hopwood *et al.*, (1987) *Methods Enzymol.*, 153:116-166 all describe vectors for use in various *Streptomyces* hosts.

In a preferred embodiment, *Streptomyces* vectors are used that include sequences that allow their introduction and maintenance in *E. coli*. Such *Streptomyces/E. coli* shuttle vectors have been described (*see*, for example, Vara *et al.*, (1989) *J. Bacteriol.*,
30 171:5872-5881; Guilfoile & Hutchinson (1991) *Proc. Natl. Acad. Sci. USA*, 88: 8553-8557.)

The gene sequences, or fragments thereof, which collectively encode a PKS and/or NRPS and/or PKS/NRPS gene cluster, one or more ORFs, one or more modules, or one or more enzymatic domains of this invention, can be inserted into one or more

expression vectors, using methods known to those of skill in the art. Expression vectors will include control sequences operably linked to the desired NRPS and/or PKS coding sequence or fragment thereof. Suitable expression systems for use with the present invention include systems that function in eucaryotic and prokaryotic host cells. However, as explained above, prokaryotic systems are preferred, and in particular, systems compatible with *Streptomyces spp.* are of particular interest. Control elements for use in such systems include promoters, optionally containing operator sequences, and ribosome binding sites. Particularly useful promoters include control sequences derived from PKS and/or NRPS gene clusters, such as one or more *act* promoters. However, other bacterial promoters, such as those derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose, will also find use in the present constructs. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the beta -lactamase (*bla*) promoter system, bacteriophage lambda PL, and T5. In addition, synthetic promoters, such as the tac promoter (U.S. Patent 4,551,433), which do not occur in nature also function in bacterial host cells. In *Streptomyces*, numerous promoters have been described including constitutive promoters, such as *ermE* and *tcmG* (Shen and Hutchinson, (1994) *J. Biol. Chem.* 269: 30726-30733), as well as controllable promoters such as *actI* and *actIII* (Pleper *et al.*, (1995) *Nature*, 378: 263-266; Pieper *et al.*, (1995) *J. Am. Chem. Soc.*, 117: 11373-11374; and Wiesmann *et al.*, (1995) *Chem. & Biol.* 2: 583-589).

Other regulatory sequences may also be desirable which allow for regulation of expression of the PKS replacement sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

Selectable markers can also be included in the recombinant expression vectors. A variety of markers are known which are useful in selecting for transformed cell lines and generally comprise a gene whose expression confers a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium. Such markers include, for example, genes that confer antibiotic resistance or sensitivity to the plasmid. Alternatively, several polyketides are naturally colored and this characteristic provides a built-in marker for selecting cells successfully transformed by the present constructs.

The various PKS and/or NRPS clusters or subunits of interest can be cloned into one or more recombinant vectors as individual cassettes, with separate control elements, or under the control of, *e.g.*, a single promoter. The PKS and/or NRPS subunits can include flanking restriction sites to allow for the easy deletion and insertion of other PKS subunits so that hybrid PKSs can be generated. The design of such unique restriction sites is known to those of skill in the art and can be accomplished using the techniques described above, such as site-directed mutagenesis and PCR.

Methods of cloning and expressing large nucleic acids such as gene clusters, including PKS- or NRPS-encoding gene clusters, in cells including *Streptomyces* are well known to those of skill in the art (*see, e.g.*, Stutzman-Engwall and Hutchinson (1989) *Proc. Natl. Acad. Sci. USA*, 86: 3135-3139; Motamedi and Hutchinson (1987) *Proc. Natl. Acad. Sci. USA*, 84: 4445-4449; Grim *et al.* (1994) *Gene*, 151: 1-10; Kao *et al.* (1994) *Science*, 265: 509-512; and Hopwood *et al.* (1987) *Meth. Enzymol.*, 153: 116-166). In some examples, nucleic acid sequences of well over 100kb have been introduced into cells, including prokaryotic cells, using vector-based methods (*see, for example*, Osoegawa *et al.*, (1998) *Genomics*, 52: 1-8; Woon *et al.*, (1998) *Genomics*, 50: 306-316; Huang *et al.*, (1996) *Nucl. Acids Res.*, 24: 4202-4209). In addition, the cloning and overexpression of NRPS-1 and NRPS-6 is illustrated in Example 1.

In certain embodiments this invention may make use of genetically engineered cells that either lack PKS and/or NRPS genes or have their naturally occurring PKS and/or NRPS genes substantially deleted. These host cells can be transformed with recombinant vectors, encoding a variety of PKS and/or NRPS gene clusters, for the production of active polyketides. The invention provides for the production of significant quantities of product, *e.g.* a bleomycin, at an appropriate stage of the growth cycle. The BLMs or other hybrid polyketide/peptide metabolites so produced can be used as therapeutic agents, to treat a number of disorders, depending on the type of metabolites in question. For example, several of the polyketides and peptides produced by the present method will find use as immunosuppressants, as anti-tumor agents, as well as for the treatment of viral, bacterial and parasitic infections. The ability to recombinantly produce polyketides and peptides also provides a powerful tool for characterizing PKSs and/or NRPSs and the mechanism of their actions.

B) Host cells.

The vectors described above can be used to express various protein components of the polyketide and/or polypeptide synthetic modules for subsequent isolation and/or to provide a biological synthesis of one or more desired biomolecules (*e.g.* polyketides, peptides, *etc.*). Where one or more proteins of the *blm* cluster are expressed (*e.g.* overexpressed) for subsequent isolation and/or characterization, the proteins are expressed in any prokaryotic or eukaryotic cell suitable for protein expression. In one preferred embodiment, the proteins are expressed in *E. coli*. Overexpression of *blmI* in *E. coli* is described in Example 2.

Host cells for the recombinant production of the subject polyketides can be derived from any organism with the capability of harboring a recombinant PKS, NRPS or PKS/NRPS gene cluster. Thus, the host cells of the present invention can be derived from either prokaryotic or eucaryotic organisms. However, preferred host cells are those constructed from the actinomycetes, a class of mycelial bacteria which are abundant producers of a number of polyketides and peptides. A particularly preferred genus for use with the present system is *Streptomyces*. Thus, for example, *S. verticillus*, *S. ambofaciens*, *S. avermitilis*, *S. azureus*, *S. cinnamonensis*, *S. coelicolor*, *S. curacoi*, *S. erythraeus*, *S. fradiae*, *S. galilaeus*, *S. glaucescens*, *S. hygrosopicus*, *S. lividans*, *S. parvulus*, *S. peucetius*, *S. rimosus*, *S. roseofulvus*, *S. thermotolerans*, *S. violaceoruber*, among others, will provide convenient host cells for the subject invention, with *S. coelicolor* being preferred (*see, e.g., Hopwood, D. A. and Sherman, D. H. Ann. Rev. Genet. (1990) 24:37-66; O'Hagan, D. The Polyketide Metabolites (Ellis Horwood Limited, 1991), for a description of various polyketide-producing organisms and their natural products.*)

In a preferred embodiment, the above-described cells are genetically engineered by deleting one or more naturally occurring PKS and/or NRPS genes therefrom, using standard techniques, such as by homologous recombination. (*see, e.g., Khosla, et al. (1992) Molec. Microbiol. 6: 3237.*)

In certain embodiments, a eukaryotic host cell is preferred (*e.g.* where certain glycosylation patterns are desired). Suitable eukaryotic host cells are well known to those of skill in the art. Such eukaryotic cells include, but are not limited to yeast cells, insect cells, plant cells, fungal cells, and various mammalian cells (*e.g.* COS, CHO HeLa cells lines and various myeloma cell lines)

C) Protein/polyketide recovery.

Polypeptide and/or polyketide recovery is accomplished according to standard methods well known to those of skill in the art. Thus, for example where *blm* cluster proteins are to be expressed and isolated, the proteins can be expressed with a convenient tag to facilitate isolation (e.g. a His₆) tag. Other standard protein purification techniques are suitable and well known to those of skill in the art (see, e.g., Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595; Nakano *et al.* (1992) *Mol. Gen. Genet.* 232: 313-321, etc.).

Similarly where components (e.g. modules and/or enzymatic domains) of the *blm* cluster are used to express various biomolecules (e.g. polyketides, sugars, polypeptides, etc.) the desired product and/or shunt metabolite(s) are isolated according to standard methods well known to those of skill in the art (see, e.g., Carreras and Khosla (1998) *supra.*) Purification and in vitro reconstitution of the essential protein components of an aromatic polyketide synthase. *Biochemistry* 37: 2084-2088, Deutscher (1990) *Methods in Enzymology Volume 182: Guide to Protein Purification*, M. Deutscher, ed. .

III. Synthesis of recombinant bleomycins.

In one embodiment this invention provides methods of synthesizing bleomycins and recombinantly synthesized bleomycins. As indicated above, this is generally accomplished by providing an organism (e.g. a bacterial cell) containing sufficient components of the *blm* gene cluster to direct synthesis of a complete bleomycin.

In one embodiment, the entire *blm* cluster is cloned into a *Streptomyces* strain (e.g., *S. lividans* or *S. coelicolor*). Kao *et al.* (1994) *Science*, 265: 509-512, have cloned the 30 kb DEBS genes from *Sacc. erythraea* into *S. coelicolor* and produced 6-deoxyerythronolide B in *S. coelicolor* and these methods can be used to construct an expression plasmid for heterologous expression of the *blm* cluster. This method involves the transfer of DNA between a temperature-sensitive plasmid and a shuttle vector by means of a homologous double recombination event in *E. coli* (*Id.*). In a preferred embodiment, the two ends spanning the *blm* cluster are cloned into a temperature-sensitive plasmid that is chloramphenicol resistant (Cm^R) such as pCK6. *S. verticillius* DNA is then rescued from a donor into the temperature-sensitive recipient by co-transforming *E. coli* with the Cm^R recipient plasmid and the apramycin resistant (Ap^R) pKC505 donor cosmid that contains the *blm* gene cluster, followed by chloramphenicol and apramycin selection at 30°C. Colonies harboring both plasmids (Cm^R, Ap^R) will be shifted to 44°C on chloramphenicol and apramycin plates and only those cointegrates formed by a single recombination event

between the two plasmids are viable. Surviving colonies are then propagated at 30°C on Cm^R plates to select for recombinant plasmids formed by the resolution of cointegrates through a second recombinant event. The desired *blm* cluster is cloned into the Cm^R temperature-sensitive plasmid and is ready to be moved into any expression plasmid by a similar means of homologous recombinant event.

For example, if pWHM861 is the choice of shuttle plasmid for the expression of the *blm* cluster in *S. lividans* (Meurer and Hutchinson (1995) *J. Bacteriol.*, 177: 477-481), the two ends spanning the *blm* cluster downstream of the *ErmE** promoter in the ampicillin resistant (AM^R) plasmid pWHM861 are cloned. The resulting plasmid is co-transformed with the temperature-sensitive plasmid containing the *blm* cluster described above into *E. coli* under the selection of chloramphenicol and ampicillin at 30°C. These Cm^R and AM^R colonies are shifted to 44°C on chloramphenicol and ampicillin plates to undergo a single recombination event and the surviving colonies are resolved on ampicillin plates at 30°C by completing the double recombination process. The resulting plasmid is suitable for transformation into *S. lividans* by selection of thiostrepton, in which the expression of the desired *blm* cluster is under the control of the *ErmE** promoter. The *S. lividans* transformants are cultured and any metabolites produced are isolated and characterized.

Once production of BLM in *S. lividans* is established, mutated alleles of the *blm* synthetase can be introduced into the *blm* cluster for the production of BLM analogs.

IV. Altered endogenous expression of bleomycins.

Using the Blm gene cluster information provided herein, one of skill in the art may regulating the synthesis of endogenous bleomycin. The expression of various ORFs comprising the *blm* gene cluster may be increased or decreased to alter bleomycin synthesis levels.

Methods of altering the expression of endogenous genes are well known to those of skill in the art. Typically such methods involve altering or replacing all or a portion of the regulatory sequences controlling expression of the particular gene that is to be regulated. In a preferred embodiment, the regulatory sequences (*e.g.*, the native promoter) upstream of one or more of the *blm* ORFs are altered.

This is typically accomplished by the use of homologous recombination to introduce a heterologous nucleic acid into the native regulatory sequences. To downregulate expression of one or more *blm* ORFs, simple mutations that either alter the reading frame or disrupt the promoter are suitable. To upregulate expression of the *blm* ORF(s) the native

promoter(s) can be substituted with heterologous promoter(s) that induce higher than normal levels of transcription.

In a particularly preferred embodiment, nucleic acid sequences comprising the structural gene in question or upstream sequences are utilized for targeting heterologous recombination constructs.

The use of homologous recombination to alter expression of endogenous genes is described in detail in U.S. Patent 5,272,071, WO 91/09955, WO 93/09222, WO 96/29411, WO 95/31560, and WO 91/12650.

V. Synthesis of BLM analogs.

In one embodiment, this invention provides methods of synthesizing modified bleomycins or bleomycin analogs. In preferred embodiments, the BLM analogs are synthesized either by introducing specific perturbations into individual NRPS and/or PKS enzymatic domains or modules, or by reprogramming the linear order in which the NRPS or PKS enzymatic domains and/or modules appear in the *blm* synthetase genes. The former will lead to BLM analogs with targeted modifications at the BLM backbone and the latter will allow incorporation of other extension units in variable sequence into the biosynthesis of BLM. In particularly preferred embodiments, the genetically modified *blm* synthetases are produced in *S. verticillus*, however, it will be recognized that the entire *blm* gene cluster can be cloned into other hosts, *e.g.* into *S. lividans* or *S. coelicolor*.

In preferred embodiments modification of the *blm* gene cluster to yield BLM analogues is accomplished by one of two different approaches. In one approach, the BLM enzymatic domains and/or modules are altered in a directed manner (*i.e.* they are changed in a preselected way), while in another approach, random/haphazard alterations are introduced into the *blm* cluster and the resulting products are screened to identify those with desired properties.

A) Synthesis of BLM analogs by specific engineering of the *blm* synthetase genes.

The *blm* synthetase genes can be re-engineered by means of specific mutations or by reprogramming the linear order of the NRPS or PKS enzymatic domains or modules. In this approach, a wild-type *blm* synthetase allele is replaced with these mutants in and expressed in an appropriate host (*e.g.*, *S. verticillus* or in a heterologous host). Since both NRPSs (Stachelhaus *et al.* (1995) *Science*, 269: 69-72) and PKSs (Donadio *et al.* (1993)

Proc. Natl. Acad. Sci. USA, 90: 7119-7123, Donadio *et al.* (1995) *J. Am., Chem. Soc.*, 117: 9105-9106, Cortes *et al.* (1995) *Science*, 268: 1487-1489) have shown considerable tolerance to reprogramming, it is expected that these modifications of the BLM synthetase will result in the production of BLM analogs with predicted structural alterations. For example, targeted modification at the (2S,3S,4R)-4-amino-3-hydroxy-2-methyl/pentanoic acid AHM moiety of BLM can be accomplished by introduction of mutations into the *BLMVIII* PKS module of the BLM synthetase locus. Inactivation of the MT or KR motif by in-frame deletion or site-directed mutagenesis will result in the production of BLM analogs containing a demethyl-AHM, oxo-AHM, or oxo-demethyl-AHM moiety, *etc.*

Alternatively, individual functional NRPS domains and/or the PKS module can be deleted or the PKS module can be duplicated in-frame to produce BLM analogs with shorter or longer backbone, respectively. Alternatively, or in addition, the NRPS domains or the PKS module can be rearranged for the production of BLM analogs with a completely different backbone. The NRPS and PKS features can be combined into one integrated system, providing access to a structural variation not available by either the NRPS or PKS system alone.

To create such mutations, plasmids are constructed carrying in-frame deletions of DNA segments encompassing a portion of the *blm* synthetase activities. Construction of specific deletions is preferably accomplished by one of the following two strategies. The first involves subcloning of a DNA fragment in a gene replacement vector, selection of two restriction sites suitably located at the two ends of the DNA segments, and deletion of this segment from within the plasmid by rejoining the two resulting ends. An in-frame deletion can be obtained by a suitable combination of Klenow filling and S1 treatment of both ends prior to ligation.

The second approach involves polymerase chain reaction (PCR) amplification of two DNA segments that separate the region to be deleted followed by joining of the two fragments in the correct orientation in a gene replacement vector. This can be accomplished by designing PCR primers with suitable restriction sites. The restriction site used to generate the deletion and the sequences to serve as templates for the PCR amplification are chosen so as to generate two segments of *blm* synthetase DNA of approximately equal length in the construction in order to maximize the chance of gene replacement. The gene replacement vector containing the allelic or deletion mutation is introduced into a *Streptomyces* strain (*e.g.*, *S. verticillus*). Integration of the plasmid into the *S. verticillus* chromosome via a single reciprocal homologous recombination will yield a recombinant that will be isolated by

selection for the vector marker. The resulting integrants are then grown under non-selective conditions and further resolution by selection for the loss of the vector marker via the second homologous recombination event will produce the desired deletion mutants.

Southern analysis of the isolated deletion mutants with the target DNA is performed to ensure that the expected double crossover recombination event has taken place. The first approach is convenient if there are suitably spaced restriction sites in the DNA sequence. The second approach enables the deletion of any DNA segment but may be limited by the size of the DNA segments that can be amplified by PCR. These *S. verticillus* recombinants are cultured under typical conditions for BLM production and the fermentation broth is screened for the production of any novel BLM analogs resulted from the specific mutations in the *blm* synthetase locus.

B) Synthesis of BLM analogs by "random" modification of *blm* synthetase genes.

Bleomycin analogs can also be synthesized by randomly/haphazardly altering genes in the BLM cluster expressing the products of the randomly modified megasynthetase and then screening the products for the desired activity. Methods of "randomly" altering *blm* cluster genes are described below.

VI. Generation of other synthetic systems.

In addition to the production of bleomycin or modified bleomycins, the *blm* gene cluster or elements thereof can be used by themselves or in combination with NRPS and/or PKS modules and/or enzymatic domains of other PKS and/or NRPS systems to produce a wide variety of compounds including, but not limited to various polyketides, polypeptides, polyketide/polypeptide hybrids, various oxazoles and thiazoles, various sugars, various methylated polypeptides/polyketides, and the like. As with the production of modified bleomycins described above, such compounds can be produced, *in vivo* or *in vitro*, by catalytic biosynthesis using large, modular PKSs, NRPSs, and hybrid PKS/NRPS systems. The megasynthetases directing such syntheses can be rationally designed *e.g.* by predetermined alteration/modification of polyketide and/or polypeptide and/or hybrid PKS/NRPS pathways. Alternatively, large combinatorial libraries of cells harboring various megasynthetases can be produced by the random modification of particular pathways and then selected for the production of a molecule or molecules of interest. It will be appreciated that, in certain embodiments, such libraries of megasynthetases/modified pathways, can be

used to generate large, complex combinatorial libraries of compounds which themselves can be screened for a desired activity.

A) Directed modification of biomolecules.

Elements (e.g. open reading frames) of the *blm* biosynthetic gene cluster and/or variants thereof can be used in a wide variety of "directed" biosynthetic processes (i.e. where the process is designed to modify and/or synthesize one or more particular preselected metabolite(s)). Polypeptides encoded by particular open reading frames or combinations of open reading frames can be utilized to perform particular chemical modifications of biological molecules.

Thus, for example, open reading frames encoding a polypeptide synthetase can be used to chemically modify an amino acid by coupling it to another amino acid. In another example, the methyl transferase in *BlmVIII* can be utilized to introduce methyl groups into polyketides, and other, substrates. The glycosyl transferases can be used to glycosylate appropriate substrates, and so forth. These examples, are merely illustrative. One of skill in the art, utilizing the information provided here, can perform literally countless chemical modifications and/or syntheses using either "native" bleomycin biosynthesis metabolites as the substrate molecule, or other molecules capable of acting as substrates for the particular enzymes in question. Other substrates can be identified by routine screening. Methods of screening enzymes for specific activity against particular substrates are well known to those of skill in the art.

The biosyntheses can be performed *in vivo*, e.g. by providing a host cell comprising the desired *blm* gene cluster open reading frame(s) and/or *in vivo*, e.g., by providing the polypeptides encoded by the *blm* gene cluster ORFs and the appropriate substrates and/or cofactors.

B) Directed engineering of novel synthetic pathways.

In numerous embodiments of this invention, novel polyketides, polypeptides, and combinations thereof are created by modifying known PKSs or NRPSs so as to introduce variations into known polymers synthesized by the enzymes. Such variations may be introduced by design, for example to modify a known molecule in a specific way, e.g. by replacing a single monomeric unit within a polymer with another, thereby creating a derivative molecule of predicted structure. Such variations can also be made by adding one or more modules to a known PKS or NRPS, or by removing one or more module from a

known PKS or NRPS. Such novel PKSs or NRPSs can readily be made using a variety of techniques, including recombinant methods and *in vitro* synthetic methods.

Using any of these methods, it is possible to introduce PKS domains into a NRPS, or vice versa, thereby creating novel molecules including both peptide and polyketide structural domains. For example, a PKS enzyme producing a known polyketide can be modified so as to include an additional module that adds a peptide moiety into the polyketide. Novel molecules synthesized using these methods can be screened, using standard methods, for any activity of interest, such as antibiotic activity, effects on the cell cycle, effects on the cytoskeleton, etc.

Novel polyketides, polypeptides, or combinations thereof can also be made by creating novel PKSs or NRPSs *de novo*, using recombinant or *in vitro* synthetic methods. Such novel arrangements of domains can be designed, *i.e.* to create a specific polymer. In addition to creating novel PKSs or NRPSs by combining modules, the methods of this invention can also be used to make novel modules that can add new monomeric units to a growing polypeptide or polyketide chain. Because the identity of each module, and, consequently, the identity of the monomer added by the module, is determined by the identity and number of the functional domains comprising the module, it is possible to produce novel monomeric units by creating novel combinations of functional domains within a module. Such novel modules can be created by design, for example to make a specific module that will add a specific monomer to a polyketide or polypeptide, or can be created by the random association of domains so as to produce libraries of novel modules. Such novel modules can be made using recombinant or *in vitro* synthetic means.

Mutations can be made to the native NRPS and/or PKS subunit sequences and such mutants used in place of the native sequence, so long as the mutants are able to function with other PKS and/or PKS subunits to collectively catalyze the synthesis of an identifiable polyketide and/or polypeptide. Such mutations can be made to the native sequences using conventional techniques such as by preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding a NRPS and/or PKS subunit using restriction endonuclease digestion. (*see, e.g., Kunkel, (1985) Proc. Natl. Acad. Sci. USA 82: 448; Geisselsoder et al. (1987) BioTechniques 5: 786*). Alternatively, the mutations can be effected using a mismatched primer (generally 10-20 nucleotides in length) which hybridizes to the native nucleotide sequence, at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by keeping the mutant base

centrally located (Zoller and Smith (1983) *Meth, Enzymol.* 100: 468). Primer extension is effected using DNA polymerase, the product cloned and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Selection can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations (*see, e.g., Dalbie-McFarland et al. (1982) Proc. Natl. Acad. Sci USA 79:6409*). PCR mutagenesis will also find use for effecting the desired mutations.

C) Random modification of PKS/NRPS pathways.

In another embodiment, variations can be made randomly, for example by making a library of molecular variants of a known polymer by randomly mutating one or more PKS or NRPS modules and/or enzymatic domains or by randomly replacing one or more modules or enzymatic domains in a known PKS or NRPS with a collection of alternative modules and/or enzymatic domains..

The PKS and/or NRPS modules can be combined into a single multi-modular enzyme, thereby dramatically increasing the number of possible combinations obtained using these methods. These combinations can be made using standard recombinant or nucleic acid amplification methods, for example by shuffling nucleic acid sequences encoding various modules or enzymatic domains to create novel arrangements of the sequences, analogous to DNA shuffling methods described in Cramer *et al.*, (1998) *Nature* 391: 288-291, and in U.S. Patents 5,605,793 and in 5,837,458. In addition, novel combinations can be made *in vitro*, for example by combinatorial synthetic methods. Novel polymers, or polymer libraries, can be screened for any specific activity using standard methods.

Random mutagenesis of the nucleotide sequences obtained as described above can be accomplished by several different techniques known in the art, such as by altering sequences within restriction endonuclease sites, inserting an oligonucleotide linker randomly into a plasmid, by irradiation with X-rays or ultraviolet light, by incorporating incorrect nucleotides during *in vitro* DNA synthesis, by error-prone PCR mutagenesis, by preparing synthetic mutants or by damaging plasmid DNA *in vitro* with chemicals. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, agents which damage or remove bases thereby preventing normal base-pairing such as hydrazine or formic acid, analogues of nucleotide precursors such as nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine intercalating agents such as proflavine, acriflavine, quinacrine, and the like.

Generally, plasmid DNA or DNA fragments are treated with chemicals, transformed into *E. coli* and propagated as a pool or library of mutant plasmids.

Large populations of random enzyme variants can be constructed *in vivo* using "recombination-enhanced mutagenesis." This method employs two or more pools of, for example, 10^6 mutants each of the wild-type encoding nucleotide sequence that are generated using any convenient mutagenesis technique, described more fully above, and then inserted into cloning vectors.

D) Incorporation and/or modification of non-blm cluster elements.

In either the directed or random approaches, nucleic acids encoding novel combinations of modules and/or enzymatic are introduced into a cell. In one embodiment, nucleic acids encoding one or more PKS or NRPS domains are introduced into a cell so as to replace one or more domains of an endogenous PKS or NRPS within a chromosome of the cell. Endogenous gene replacement can be accomplished using standard methods, such as homologous recombination. Nucleic acids encoding an entire PKS, NRPS, or combination thereof can also be introduced into a cell so as to enable the cell to produce the novel enzyme, and, consequently, synthesize the novel polymer. In a preferred embodiment, such nucleic acids are introduced into the cell optionally along with a number of additional genes, together called a 'gene cluster,' that influence the expression of the genes, survival of the expressing cells, *etc.* In a particularly preferred embodiment, such cells do not have any other PKS- or NRPS- encoding genes or gene clusters, thereby allowing the straightforward isolation of the polymer synthesized by the genes introduced into the cell.

Furthermore, the recombinant vector(s) can include genes from a single PKS and/or NRPS gene cluster, or may comprise hybrid replacement PKS gene clusters with, *e.g.*, a gene for one cluster replaced by the corresponding gene from another gene cluster. For example, it has been found that ACPs are readily interchangeable among different synthases without an effect on product structure. Furthermore, a given KR can recognize and reduce polyketide chains of different chain lengths. Accordingly, these genes are freely interchangeable in the constructs described herein. Thus, the replacement clusters of the present invention can be derived from any combination of PKS and/or NRPS gene sets that ultimately function to produce an identifiable polyketide and/or peptide.

Examples of hybrid replacement clusters include, but are not limited to, clusters with genes derived from two or more of the *act* gene cluster, the *whiE* gene cluster, frenolicin (*fren*), granaticin (*gra*), tetracenomycin (*tcm*), 6-methylsalicylic acid (6-msas),

oxytetracycline (*otc*), tetracycline (*tet*), erythromycin (*ery*), griseusin (*gris*), nanaomycin, medermycin, daunorubicin, tylosin, carbomycin, spiramycin, avermectin, monensin, nonactin, curamycin, rifamycin and candicidin synthase gene clusters, among others. (For a discussion of various PKSs, *see, e.g.*, Hopwood and Sherman (1990) *Ann. Rev. Genet.* 24: 37-66; O'Hagan (1991) *The Polyketide Metabolites*, Ellis Horwood Limited.

A number of hybrid gene clusters have been constructed, having components derived from the *act*, *fren*, *tcn*, *gris* and *gra* gene clusters (*see, e.g.*, U.S. Patent 5,712,146). Other hybrid gene clusters, as described above, can easily be produced and screened using the disclosure herein, for the production of identifiable polyketides, polypeptides or polyketide/polypeptide hybrids.

Host cells (*e.g. Streptomyces*) can be transformed with one or more vectors, collectively encoding a functional PKS/NRPS set (*e.g.* a bleomycin or bleomycin analog), or a cocktail comprising a random assortment of PKS and/or NRPS genes, modules, active sites, or portions thereof. The vector(s) can include native or hybrid combinations of PKS and/or NRPS subunits or cocktail components, or mutants thereof. As explained above, the gene cluster need not correspond to the complete native gene cluster but need only encode the necessary PKS and/or NRPS components to catalyze the production of the desired product. For example, in *Streptomyces* aromatic PKSs, carbon chain assembly requires the products of three open reading frames (ORFs). ORF1 encodes a ketosynthase (KS) and an acyltransferase (AT) active site (KS/AT); ORF2 encodes a chain length determining factor (CLF), a protein similar to the ORF1 product but lacking the KS and AT motifs; and ORF3 encodes a discrete acyl carrier protein (ACP). Some gene clusters also code for a ketoreductase (KR) and a cyclase, involved in cyclization of the nascent polyketide backbone. However, it has been found that only the KS/AT, CLF, and ACP, need be present in order to produce an identifiable polyketide. Thus, in the case of aromatic PKSs derived from *Streptomyces*, these three genes, without the other components of the native clusters, can be included in one or more recombinant vectors, to constitute a "minimal" replacement PKS gene cluster.

E) Variation of starter and extender units.

In addition to varying the PKS and/or NRPS modules and/or domains, variations in the products produced by various PKS/NRPS systems can be obtained by varying the starter units and/or the extender units. Thus, for example, a considerable degree of variability exists for starter units, *e.g.*, acetyl CoA, maloamyl CoA, propionyl CoA,

acetate, butyrate, isobutyrate and the like. In addition, naturally occurring PKSs and/or NRPSs have shown some tolerance for varying extender units.

F) Examples of preferred modifications.

As indicated above, the novel PKS and NRPS modules and enzymatic domains identified herein can be used to perform specific single modifications of particular substrates, or as components of complex synthetic pathways to generate particular products or large combinatorial libraries. As described in the Examples, a number of modules of the *blm* gene cluster provide novel functionality. By way of example, a few preferred reactions are listed below. These examples are intended to be illustrative and are not exhaustive nor limiting.

1. Use of *BlmVIII* PKS to introduce branched methyl group.

The *blmVIII* gene identified herein encodes a PKS module consisting of domains characteristic for known PKSs, such as ketoacyl synthase (KS), acyltransferase (AT), ketoreductase (KR), and ACP, with malonyl CoA acting as an extending unit.

However, the identification of an integrated methyltransferase (MT) domain in the middle of *BlmVIII* is unique, representing the first PKS from actinomycetes that contains an internal MT domain. The use of this methyltransferase domain allows the introduction of a branched methyl group during a polyketide and/or polypeptide and/or hybridizing polyketide/polypeptide synthesis. Figure 5 illustrates the use of *BlmVIII* PKS in engineering a polyketide biosynthesis that introduces a branched methyl group.

The first formula in Figure 5 illustrates a polyketide synthesis mediated by 6-deoxyerythronolide B synthase (DEBS) which normally catalyzes the biosynthesis of the erythromycin aglycone, 6-deoxyerythronolide B. The remaining formulas show how the use of the *blmVIII* methyltransferase (MT) group at different points in the synthesis results in the introduction of a methyl group at different locations in the resulting product.

In view of this illustration, one of skill in the art would appreciate that the *blmVIII* MT domain can be used in a wide variety of biosyntheses to introduce methyl branches.

2. Use of the *blm* gene cluster to make thiazolidine, thiazoline, thiazole, bi-thiazolidine, bithiazoline, and bithiazole-containing compounds.

The *BlmIV* and *BlmIII* NRPSs are characterized by unusual Cy domains as well an unprecedented Ox domain, providing an efficient biosynthesis for a bithiazole structure. While thiazoline is the direct product of the Cy domain, the thiazoline-to-thiazole conversion generally is performed with an additional oxidation step. We identified at the C-terminus of NRPS-0 an additional domain that shows low, but significant, sequence homology to a family of putative oxidases/dehydrogenases, including the McbC protein of the microcin B17 synthase (Table 1). Microcin B17 synthase catalyzes the synthesis of the oxazole and thiazole-containing peptide antibiotic microcin B17, and McbC has been proposed to play a role in catalyzing the oxazoline/thiazoline-to-oxazole/thiazole conversion. Consequently, we propose that this extra domain at the C-terminus of NRPS-0 provides the oxidase/dehydrogenase activity for the biosynthesis of the bithiazole moiety of BLM, defining a novel Ox domain for NRPSs.

It is noteworthy that a cell-free preparation from *Sv* ATCC15003 has been reported to catalyze the conversion of phleomycins to BLMs in the presence of NAD^+ , supporting the hypothesis that the bithiazole moiety of BLM results from stepwise oxidations of a bithiazoline precursor (Fig. 1A). (The phleomycin producer could be imagined to result from the loss of its Ox activity for the first thiazoline ring.) Given the wide distribution of thiazole or oxazole rings in natural products exhibiting an impressive array of biological activities, the cloning of the *blmIV*, *III* genes and the identification of the Ox domain open many opportunities thiazole biosynthesis and to synthesize novel thiazole containing molecules by engineering peptide biosynthesis.

Representative thiazole syntheses using variants of the *blm* NRPS are illustrated in Figure 6. Note that in Figure 6, A^{M} and A^{N} refer to an A domain that activates and amino acid with R^{M} and R^{N} groups, respectively. A^{C} refers to an A domain that activates Cys ($\text{x} = \text{SH}$) or Ser ($\text{X} \rightleftharpoons \text{OH}$) that can be cyclized to form the oxiazoline/thiazoline or oxazole/thiazole structures. DH is a dehydratase. In view of these representative examples, one of skill in the art would appreciate that the *blm* NRPS domain and its variants can be used in a wide variety of chemical syntheses make thiazolidine, thiazoline, thiazole, bi-thiazolidine, bithiazoline, or bithiazole-containing compounds.

3. Use of the *blm* gene cluster to make heterocyclic ring-containing compounds.

Various *blm* modules can be used to produce heterocyclic ring-containing compounds. Such heterocycles include, but are not limited to five member S- and N-
5 containing compounds of the thiazolidine, thiazoline and thiazole family or the O- and N-
containing compounds of the oxazolidine, oxazoline, and oxazole family. Again, the
preparation of such compounds is illustrated in Figure 6.

4. Use of the *blm* gene cluster to make sugars.

In still another embodiment, the *blm* gene cluster or elements thereof can be
10 used to make sugars. Such sugars include, but are not limited to L-sugars (with the *BlmG*
epimerase), sugars modified by a carbamoyl group (*e.g.*, using *BlmD*), and various
disaccharides. Representative examples of such syntheses are illustrated in Figure 7. Such
sugar biosynthesis genes can also be used to attach sugars onto other polyketide and/or
peptide aglycones.

F) Screening of products.

Particularly where large combinatorial libraries are synthesized, *e.g.* using one
or more modules and/or enzymatic domains of the *blm* gene cluster it will often be desired to
screen the resulting compound(s) for the desired activity. Methods of screening compounds
(*e.g.* polypeptides, polyketides, sugars, thiazoles, *etc.*) for various activities of interest (*e.g.*
20 cytotoxicity, antimicrobial activity, particular chemical activities, *etc.*) are well known to
those of skill in the art.

Where large numbers of compounds are produced, it is often desired to
rapidly screen such compounds using "high throughput systems" (HTS). High throughput
assays systems are well known to those of skill in the art and many such systems are
25 commercially available. (*see, e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries,
Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick,
MA, *etc.*). These systems typically automate entire procedures including all sample and
reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate
in detector(s) appropriate for the assay. These configurable systems provide high
30 throughput and rapid start up as well as a high degree of flexibility and customization. The
manufacturers of such systems typically provide detailed protocols for the various high
throughput screens.

VII. In Vitro syntheses.

In additional embodiments of this invention, bleomycins and other polyketides and/or polypeptides are synthesized and/or modified *in vitro*. Individual enzymatic domains or modules can be used *in vitro* to modify a unit and/or to add a single monomeric unit to a growing polyketide or polypeptide chain. In one approach a metasyntetase providing all the desired synthetic activities recombinantly expressed and then provided, the appropriate substrates and buffer system *e.g.* in a bioreactor, to direct the synthesis of the desired product. In another approach, various PKSs and/or NRPSs are provided in different solutions and the growing polymer chains can be sequentially introduced into the plurality of solutions, each containing a single (or several) PKS or NRPS modules. In still another embodiment, the PKS and/or NRPS modules or enzymatic domains are provided attached to a solid support and a fluid containing the growing macromolecule is passed over the surface whereby the PKSs or NRPSs are able to react with the target substrate.

In one preferred embodiment, a combinatorial library of polyketides or polypeptides, or combinations thereof, is created by using automated means to facilitate the sequential introduction of a multitude of polymeric chains, each attached to a solid support, to a collection of solutions, each containing a single PKS or NRPS module. These automated means can be used to systematically vary the sequence by which each polymeric chain is introduced into the various solutions, thereby creating a combinatorial library. Numerous methods are well known in the art to create combinatorial libraries of molecules by the sequential addition of monomeric units, for example as described in WO 97/02358.

VIII. Kits.

In still another embodiment, this invention provides kits for practice of the methods described herein. In one preferred embodiment, the kits comprise one or more containers containing nucleic acids encoding one or more of the *blm* gene cluster ORFs and/or one or more of the BLM PKS or NRPS modules or enzymatic domains. Certain kits may comprise vectors encoding the *blm* orfs and/or cells containing such vectors. The kits may optionally include any reagents and/or apparatus to facilitate practice of the assays described herein. Such reagents include, but are not limited to buffers, labels, labeled antibodies, bioreactors, cells, *etc.*

In addition, the kits may include instructional materials containing directions (*i.e.*, protocols) for the practice of the methods of this invention. Preferred instructional

materials provide protocols utilizing the kit contents for creating or modifying *blm* module or ORF and/or for synthesizing or modifying a molecule using one or more *blm* modules and/or enzymatic domains. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

Bleomycin biosynthesis in *Streptomyces verticillus* ATCC15003, A model for hybrid peptide and polyketide biosynthesis.

Here we report the cloning and characterization of the *blm* biosynthesis gene cluster from *Sv* ATCC15003 (Fig. 2). Sequence analysis and biochemical characterization of individual modules enabled us to align the nine NRPS and one PKS modules in a linear order to constitute the Blm megasynthetase complex (Fig. 1B). These studies revealed several unprecedented features for peptide and polyketide biosynthesis, setting the stage to investigate the molecular basis for intermodular communication between NRPS and PKS, and supported the wisdom of combining individual NRPS and PKS modules for combinatorial biosynthesis to make novel “unnatural” natural products from amino acids and short carboxylic acids.

Materials and Methods.

General procedures.

Escherichia coli DH5 α (Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA), *E. coli* XL 1-Blue MR (Stratagene, La Jolla, CA), *E. coli* BL21(DE-3) (Novagen, Madison, WI), and *Sv* ATCC15003 (American Type Culture Collection, Rockville, MD) were used in this work. pOJ446 (Agricultural Research Service Culture Collection, Peoria, IL), pQE60 (Qiagen, Santa Clarita, CA), pET28a and pET29a (Novagen), and other plasmids

were from commercial sources. *E. coli* (Sambrook, *supra.*) and *Sv* ATCC15003 strains (Hopwood *et al.* (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual*, The John Innes Foundation, Norwich, UK) were cultured under standard conditions.

Plasmid preparation was carried out by using commercial kits (Qiagen). Total *Sv* ATCC15003 DNA was isolated according to literature protocols (Hopwood *et al.* (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual*, The John Innes Foundation, Norwich, UK; Nagaraja *et al.* (1987) *Methods Enzymol.* 153: 166-198). Restriction enzymes and other molecular biology reagents were from commercial sources, and digestions and ligation followed standard methods (Sambrook, *supra.*). For Southern analysis, digoxigenin labelling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Automated DNA sequencing was carried out on an ABI Prism 377 DNA Sequencer (Perkin-Elmer/ABI, Foster City, CA), and this service was provided by either the DBS Automated DNA Sequencing Facility, UC Davis, or Davis Sequencing (Davis, CA). Data were analyzed by the ABI Prism Sequencing 2.1.1 software and the Genetics Computer Group (GCG) program (Madison, WI).

Cloning and sequencing of the *blm* gene cluster.

A genomic library of *Sv* ATCC15003 was constructed in pOJ446 according to literature procedures (Nagaraja *et al.* (1987) *Methods Enzymol.* 153: 166-198) and screened with probes made from both ends of the *blmAB* locus (Sugiyama *et al.* (1994) *Gene* 151: 11-16; Calcutt and Schmidt (1994) *Gene* 151: 17-21), leading to the localization of 140-kb contiguous DNA, of which 100-kb is upstream (Fig. 2) and 40-kb is downstream (data not shown) of the *blmAB* genes. Heterologous NRPS probes were amplified from *Sv* ATCC15003 by polymerase chain reaction (PCR) according to literature procedures (Turgay and Marahiel (1994) *Peptide Res.* 7: 238-241) and used to screen the entire 140-kb DNA by Southern analysis under various hybridization conditions (Shen *et al.* (1999) *Bioorg. Chem.* 27: 155-171).

Prediction of substrate specificity of NRPSs.

The nine Blm NRPS modules were compared with eighty four modules from various bacterial and fungal NRPSs available at the GenBank, including those with known or putative specificity for amino acids present in BLM. A table of overall similarities/identities

was generated by PILEUP analysis of the A3 to A6 regions, and the residues lining the substrate binding pocket by comparison with PheA (Conti *et al.* (1997) *EMBO J.* 16, 4174-4183) were determined by PILEUP/PRETTY analysis. The percentage similarities for each Blm NRPS module were plotted against the rest of the NRPS modules to display the overall sequence homology between the A3 to A6 region. Those modules that showed significantly higher homology were selected to compare the amino acid residues that line the substrate binding pocket.

Overproduction and biochemical characterization of the NRPS-1A and NRPS-6A proteins.

Heterologous expression of the A domain in *E. coli* were performed according to literature procedures (Mootz and Marahiel (1997) *J. Bacteriol.* 179: 6843-6850). NRPS-1A (forward primer 5'-AAC CCA TGG CTG CTT CCC TGA CCC GCC TGG CC-3' (SEQ ID NO:76) and reverse primer 5'-CCT AGA TCT ACG GGC AGG TGG GGC GGT-3' (SEQ ID NO:77) and NRPS-6A (forward primer 5'-GGG AAT TCC ATA TGA TCC TCA CGT CCT TCC AC-3' (SEQ ID NO:78) and reverse primer 5'-GGC AAG CTT GGG TGA GGG TCC GTT CGG T-3' (SEQ ID NO:79) were amplified by PCR from *Sv* ATCC15003 cosmid clones. The resulting 1.6-kb fragment of NRPS-1A was first cloned into the *NcoI/BglIII* sites of pQE60 and then moved as an *NcoI/HindIII* fragment into the similar sites of pET29a to yield pBS10, and the resulting 1.6-kb fragment of NRPS-6A was directly cloned into the *NdeI/HindIII* sites of pET28a to yield pBS11. Introduction of pBS10 and pBS11 into *E. coli* BL21(DE-3) under standard expression conditions resulted in production of NRPS-1A (with an N-terminal S-tag and a C-terminal His₆-tag) and NRPS-6A (with an N-terminal His₆-tag), respectively. The soluble fractions of fusion proteins were subjected sequentially to an affinity chromatography on Ni-NTA resin and an anion exchange chromatography on a Hyper-D column (PerSeptive Biosystem, Framingham, MA), resulting in NRPS-1A and NRPS-6A with near homogeneity.

Results and Discussion.

Cloning of the *blm* gene cluster from *Sv* ATCC15003.

Davies and co-workers previously cloned two BLM resistance genes (*blmA* and *blmB*) from *Sv* ATCC15003 (Sugiyama *et al.* (1994) *Gene* 151: 11-16), and Calcutt and Schmidt (1994) *Gene*, 151: 17-21, sequenced a 7.2-kb DNA fragment flanking the *blmAB*

genes, revealing seven open reading frames (orfs), none of which were found to encode Blm NRPS or PKS enzymes. Given the precedent that antibiotic production genes commonly occur as a cluster in actinomycetes, we adopted an approach combining chromosomal walking from the *blmAB* resistance locus and DNA hybridization with heterologous NRPS probes to clone and identify the *blm* cluster, leading to the localization of 140-kb contiguous *Sv* ATCC15003 DNA. DNA sequencing of approximately 90-kb of the *blm* gene cluster, including the 7.2-kb *blmAB* locus, revealed 40 ORFs (Fig. 2). Preliminary functional assignments were made by comparison of the deduced gene products with proteins of known functions in the database. Among the ORFs identified from the *blm* cluster, we indeed found a PKS module, flanked by several NRPS modules—a fact that supports the hybrid NRPS/PKS/NRPS hypothesis for BLM biosynthesis—along with several sugar biosynthesis genes and genes encoding other biosynthesis enzymes as well as several resistance and regulatory genes (Table 1).

Noteworthy are the genes encoding the putative NRPS and PKS enzymes. The *blmI*, *blmII*, and *blmXI* genes encode NRPSs with an unusual architecture. In contrast to all known NRPSs, which are of modular organization with each module consisting minimally of a condensation (C), an adenylation (A), and a peptidyl carrier protein (PCP) domain (1), BlmI, BlmII, and BlmXI are discrete proteins homologous to individual domains of type I NRPSs. We have characterized BlmI as a type II PCP (18). The BlmII and BlmXI proteins could serve as candidates for type II condensation enzymes. It is unclear yet what role if any these discrete NRPS enzymes could play in BLM biosynthesis.

The *blmIII*, *blmIV*, *blmV*, *blmVI*, *blmVII*, *blmIX*, and *blmX* genes encode modular NRPSs consisting of domains characteristic for known type I NRPSs (A special thematic issue on polyketide and nonribosomal polypeptide biosynthesis, (1997) *Chem. Rev.* 97: 2463-2706), such as the A, PCP, C, and condensation/cyclization (Cy) domains (Konz *et al.* (1997) *Chem. Biol.* 4: 927-937), as well as an unprecedented oxidation (Ox) domain (see discussion below). However, BlmVI is unique among all the Blm NRPSs identified. Its N-terminal module (NRPS-5) consists of an atypical A domain, which bears a close resemblance to a family of acyl CoA synthases (Fitzmaurice and Kolattukudy (1997) *J. Bacteriol.* 179: 2608-2615; Fitzmaurice and Kolattukudy (1998) *J. Biol. Chem.* 273: 8033-8039), and an acyl carrier protein (ACP)-like domain (A special thematic issue on polyketide and nonribosomal polypeptide biosynthesis, (1997) *Chem. Rev.* 97: 2463-2706). Its C-terminal module is truncated and presumably interacts with BlmV to constitute the complete NRPS-3 module (Fig. 1B). Also noteworthy are the C domain of NRPS-3 that lacks both

His residues of the conserved HHxxxDG (SEQ ID NO:4) active site for transpeptidation (Stachelhaus *et al.* (1998) *J. Biol. Chem.*, 273: 22773-22781) and the extra C domain at the C-terminus of BlmV. These unusual features associated with *BlmVI* and *BlmV* may play roles in the formation of the β -aminoalaninamide and the pyrimidine moieties of BLM, which are unprecedented in peptide biosynthesis. For example, we propose that the NRPS-4-activated Ser is first dehydrated into dehydroalanine before condensation—an analogous Thr-to-2,3-dehydroaminobutyric acid dehydration has been observed in syringomycin biosynthesis (Guenzi *et al.* (1998) *J. Biol. Chem.* 273: 32857-32863). Conjugate addition to dehydroalanine by Asn on the NRPS-3 module downstream followed by an aminolysis to cleave the Ser-Asn adduct off the Blm megasynthetase furnishes the β -aminoalaninamide moiety (Fig. 1B). The former reaction could be catalyzed by the C domain of NRPS-3 that apparently is nonfunctional for normal transpeptidation due to the lack of the active sites, and the latter reaction could be catalyzed by the acyl CoA synthase-like domain of NRPS-5 in a process that resembles the acyl CoA synthase-catalyzed synthesis of acyl CoA from carboxylic acid (Stachelhaus *et al.* (1998) *J. Biol. Chem.* 273: 22773-22781; Guenzi *et al.* (1998) *J. Biol. Chem.* 273: 32857-32863) but in the reverse direction in the presence of an amino donor (Fig. 1B).

The *blmVIII* gene encodes a PKS module consisting of domains characteristic for known PKSs, such as ketoacyl synthase (KS), acyltransferase (AT), ketoreductase (KR), and ACP, with malonyl CoA acting as an extending unit according to sequence comparison of the AT domain (Haydock *et al.* (1995) *FEBS Lett.* 374: 246-248) (Fig. 1B). However, the identification of an integrated methyltransferase (MT) domain (Kagan and Clarke (1994) *Arch. Biochem. Biophys.* 310: 417-427) in the middle of *BlmVIII* is unique, representing the first PKS from actinomycetes that contains an internal MT domain. The only other example of PKS from bacteria that contains an internal MT domain is HMWP1 of the yersiniabactin gene cluster (Pelludat *et al.* (1998) *J. Bacteriol.* 180: 538-546). It has been assumed that fungal PKSs in general contain internal MTs for the introduction of methyl branch into the polyketide products, as it has been shown recently in lovastatin biosynthesis (Kennedy *et al.* (1999) *Science* 284: 1368-1372).

The Blm megasynthetase-templated assembly of BLM.

According to the hybrid NRPS/PKS/NRPS model for BLM biosynthesis (Fig. 1A), we predict a linear modular organization of individual NRPS and PKS modules to constitute the Blm megasynthetase. Thus, the first functional domain of the Blm

megasynthetase should be a NRPS module that initiates BLM biosynthesis by activating L-Ser as an amino acylthioester to set the stage for transpeptidation. Chain elongation proceeds by sequential incorporation of L-Asn, L-Asn, L-His, and L-Ala, requiring four additional NRPS modules. In the next step, a malonate reacts with the resulting pentapeptide intermediate to form a β -ketothioester intermediate that is subsequently methylated at the α -position and reduced at the β -keto group. A PKS module presumably dictates all these biosynthetic events and interacts with the aligned NRPS module upstream to channel the growing peptide intermediate from an NRPS module to a PKS module. After one cycle of polyketide elongation, peptide elongation is resumed by incorporation of an L-Thr residue. This step is presumably catalyzed by an NRPS module that interacts with the upstream PKS module to channel the growing polyketide intermediate (as far as the active site is concerned) from a PKS module to an NRPS module. At this stage, methylation occurs at the pyrimidine moiety of the growing intermediate, presumably catalyzed by a discrete methyltransferase; chain elongation is continued by three additional NRPS modules that incorporate a β -Ala and two L-Cys molecules sequentially. Finally, the fully assembled BLM peptide/polyketide/peptide backbone is hydroxylated at the β -position of the His residue, presumably by a discrete hydroxylase, and released from the Blm megasynthetase complex via nucleophilic substitution of the RCO-S-PCP species by a terminal amine to form the BLM aglycone. Intermediates after five of the nine proposed elongation steps were in fact isolated as P-3, P-3A, P-3K, P-4, P-5, P-5m, P-6m, and P-6mo (Takita and Muroka (1990) pages 289-309 in *Biochemistry of Peptide Antibiotics: Recent Advances in the Biotechnology of β -Lactams and Microbial Peptides*, Kleinkauf, H. & von Döhren, H. eds., W. de Gruyter, N.Y.), which presumably resulted from premature departure from the Blm megasynthetase complex before the chain reaches its full length (Fig. 1B).

Most of the bacterial NRPS gene clusters characterized to date are organized in operon-type structures, encoding multimodular NRPS proteins with individual modules organized along the chromosome in a linear order that parallels the order of the amino acids in the resultant peptides, i.e., following the “colinearity rule” for the NRPS-templated assembly of peptides from amino acids (A special thematic issue on polyketide and nonribosomal polypeptide biosynthesis, (1997) *Chem. Rev.* 97: 2463-2706; Cane *et al.* (1998) *Science* 282: 63-68). Inspection of the *blm* gene cluster (Fig. 2) showed that the Blm NRPS and PKS modules apparently are not organized according to the “colinearity rule” for BLM biosynthesis (Fig. 1). [Exception to the “colinearity rule” was also noted in the

syringomycin synthetase gene cluster (Guenzi *et al.* (1998) *J. Biol. Chem.* 273: 32857-32863), and in fact, Grandi and co-workers have demonstrated recently in *Bacillus subtilis* that neither the operon-type structure nor the physical linkage of individual modules is essential for proper assembly and activity of the surfactin NRPS megasynthetase (Guenzi *et al.* (1998) *J. Biol. Chem.* 273: 14403-14410).] Realizing that the BLM biosynthesis cannot be rationalized according to the “colinearity rule”, we determined the substrate specificity of individual NRPS and PKS modules in an attempt to shed light on the modular organization of the Blm megasynthetase complex. Brick and co-workers postulated, based on the X-ray structural analysis of the A domain of GrsA, PheA, that the region between core sequences A3 to A6 represent the amino acid specificity determinant of an NRPS module (Conti *et al.* (1997) *EMBO J.* 16: 4174-4183). Since the A domains in all known NRPSs share a significant sequence identity (ensuring that the main chain conformation of the enzymes is likely to be very similar), they further proposed that the differing substrate specificity of individual NRPS modules will be mainly determined by the nature of the amino acids lining the substrate binding pocket (Stachelhaus *et al.* (1999) *Chem. Biol.* 6: 493-505; Conti *et al.* (1997) *EMBO J.* 16: 4174-4183). Given this structural information and the vast amount of NRPS sequences available at the GenBank, we developed a novel approach for predicting substrate specificity for NRPS modules by comparing the overall sequence between the A3 to A6 region and the eight amino acid residues that line up the substrate binding pocket. While a constant level of similarities (30%-40%) was evident among all the NRPS modules analyzed, most of the Blm NRPS modules showed striking similarities (50%-60%) to a particular cluster of NRPS modules as exemplified in Fig. 3A for NRPS-1 and NRPS-6. Close examination of these modules clustered with higher similarities revealed that they activate the same or very similar amino acid, based on which the putative substrate for the NRPS in query could be predicted, i.e., NRPS-1 and NRPS-6A activate L-Cys and L-Thr, respectively. These predictions were further supported by comparing the residues lining the substrate binding pocket. For example, the amino acid residues lining the substrate binding pocket for NRPS-1 and NRPS-6 are almost identical to those NRPS modules that are known to activate L-Cys and L-Thr, respectively, as shown in Fig. 3B. To verify the predicted amino acid specificity, we overproduced and purified the NRPS-1A and NRPS-6A proteins (Fig. 3C) and examined their substrate specificity according to the amino acid-dependent ATP-PPi assay (Lee *et al.* (1970) *Meth. Enzymol.*, 43: 585-602; Ku *et al.* (1997) *Chem. & Biol.*, 4: 203-207). NRPS-1A and NRPS-6A indeed activate specifically L-Cys and L-Thr, respectively, among the amino acids tested (Fig. 3D). The latter results greatly enhanced our

confidence in predicting the substrate specificity of a NRPS module by the above method. We subsequently determined the substrate specificity for all the NRPS modules identified from the *blm* gene cluster and they in fact accounted for all nine amino acids required for BLM biosynthesis (Fig. 2).

Using the substrate specificity of individual NRPS and PKS modules as a guide, we can align the nine NRPS and one PKS modules to constitute the Blm megasynthetase as shown in Fig. 1B according to our hybrid NRPS/PKS/NRPS model for BLM biosynthesis (Fig. 1A). Among all the PKSs or NRPS systems examined so far, the Blm megasynthetase consists of the largest number of individual proteins. The precise interactions among all the Blm NRPS and Blm PKS proteins to constitute the Blm megasynthetase complex, therefore, reflect a remarkable power of protein-protein recognition (Guenzi *et al.* (1998) *J. Biol. Chem.* 273: 14403-14410; Gokhale *et al.* (1999) *Science* 284: 482-485). Although we are yet to provide direct evidence supporting the specific protein-protein interactions between the neighboring proteins, it is striking to note that all the biosynthetic intermediates isolated are derailed from either PKS or NRPS modules at the junctions between the interacting proteins (Fig. 1B). Since it is not difficult to imagine that an intermediate is more likely to fall off the enzyme complex when it is subjected to interpeptide transfer than to intrapeptide transfer, we view the latter observation as strong evidence supporting the current model of the Blm megasynthetase

BlmIX/BlmVIII/BlmVII as a hybrid NRPS/PKS/NRPS model.

Recent biosynthetic studies on rapamycin in *Streptomyces hygroscopicus* (Konig *et al.* (1997) *Eur. J. Biochem.* 247: 526-534), yersiniabactin in *Yersinia enterocolitica* and *Y. pestis* (Pelludat *et al.* (1998) *J. Bacteriol.* 180: 538-546; Gehring *et al.* (1998) *Chem. Biol.* 5: 573-586; Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650) and TA in *Myxococcus xanthus* (Paitan *et al.* (1999) *J. Mol. Biol.* 286, 465-474) are starting to shed light on hybrid peptide and polyketide biosynthesis. Two models are emerging for the alignment between a NRPS and a PKS module. The interacting NRPS and PKS modules could be either covalently linked by arranging all domains in a linear order on the same protein (Pelludat *et al.* (1998) *J. Bacteriol.* 180: 538-546; Gehring *et al.* (1998) *Chem. Biol.* 5: 573-586; Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650; Paitan *et al.* (1999) *J. Mol. Biol.* 286: 465-474) or physically located on two separate proteins, requiring specific protein-protein recognition to ensure the correct pairing between the interacting modules (Pelludat *et al.* (1998) *J. Bacteriol.* 180: 538-546; König *et al.* (1997) *Eur. J. Biochem.* 247: 526-534;

Gehring *et al.* (1998) *Chem. Biol.* 5: 573-586; Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650). Common to all these systems, however, are the unusual features associated with the interacting modules, such as the lack of the AT domain of the PKS module in Ta1 (Paitan *et al.* (1999) *J. Mol. Biol.* 286: 465-474) and the lack of the A domain and the presence of the Cy domain of the NRPS modules in both HMWP1 and HMWP2 (Pelludat *et al.* (1998) *J. Bacteriol.* 180: 538-546; Gehring *et al.* (1998) *Chem. Biol.* 5: 573-586; Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650). While extremely intriguing, the latter features complicate mechanistic analysis of these systems, making them less ideal candidates for studying how NRPS and PKS integrate into a productive hybrid NRPS/PKS complex.

The *BlmIX/BlmVIII/BlmVII* system combines the features of both hybrid NRPS/PKS and PKS/NRPS systems, serving as an ideal model for studying hybrid peptide and polyketide biosynthesis. The fact that both the *BlmIX* and *BlmVII* NRPS modules and the *BlmVIII* PKS module themselves are three separate proteins with a typical domain organization for NRPS and PKS enzymes greatly simplifies the mechanistic analysis of the hybrid NRPS/PKS/NRPS complex. We have found that the KS domain of *BlmVIII* is more similar to the KSs of HMWP1 (Pelludat *et al.* (1998) *J. Bacteriol.* 180: 538-546) and Ta1 (Paitan *et al.* (1999) *J. Mol. Biol.* 286: 465-474), both of which catalyze the elongation of a peptidyl intermediate with a malonate, than to KSs of type I PKSs. We attribute these subtle differences to their unique reactivity that catalyzes the transfer of the peptidyl intermediate from the PCP to the KS domain, which presumably takes place prior to chain elongation (Fig.4). Subsequent condensation catalyzed by the KS domain between the peptidyl intermediate and malonyl-S-ACP results in the elongation of the growing peptide with a carboxylic acid. Equally striking are the discoveries that the ACP domain of *BlmVIII* is more similar to a PCP than to an ACP and that the C domain of *BlmVII* has an additional N-terminal segment of about 50 amino acids that is rich in arginine, aspartic acid, and glutamic acid. The latter feature is analogous to the N-terminal interpolypeptide linker for type I PKS, which has recently been demonstrated to play a critical role in intermodular communication (Gokhale *et al.* (1999) *Science* 284: 482-485). We propose that these unique features of the ACP domain from the *BlmVIII* PKS module and the C domain from the *BlmVII* NRPS module provide the molecular basis for the C domain to recognize the acyl-S-ACP as a substrate. Subsequent condensation catalyzed by the C domain between acyl-S-ACP and amino acyl-S-PCP results in the elongation of the growing polyketide (as far as this condensation is concerned) with an amino acid (Fig. 4).

Novel domains for the Blm NRPS and PKS modules.

Various NRPS and PKS domains have been characterized, which are the building blocks for the entire field of combinatorial biosynthesis. The success for combinatorial biosynthesis depends critically upon the repertoire of these individual domains. Genetic analysis of the *blm* gene cluster has uncovered several novel NRPS and PKS domains. Without being bound to a particular theory, it is believed that *BlmVI* and *BlmV* are involved in the biosynthesis of the β -aminoalaninamide and pyrimidine moieties of BLM). In addition, the MT domain in *BlmVIII*, the Cy domains in *BlmIV*, and the Ox domain in *BlmIII* are novel domains.

The *BlmVIII* PKS module apparently furnishes the "propionate" unit into BLM in two steps by evolving a malonyl CoA-specifying AT domain coupled with a novel S-adenosylmethionine-requiring MT domain, representing a new mechanism to introduce methyl branches into polyketides (Fig. 4). This biosynthetic reaction sequence is unprecedented for polyketide biosynthesis since all PKSs from actinomycetes examined to date incorporate the alkyl branches into the resultant polyketides by selecting various alkyl malonates as the extending units that are determined by the AT domains. Yet, feeding experiments have unambiguously established that the polyketide moiety of BLM was derived from an acetate and a methionine (Takita and Muroka (1990) pages 289-309 in *Biochemistry of Peptide Antibiotics: Recent Advances in the Biotechnology of β -Lactams and Microbial Peptides*, Kleinkauf, H. & von Döhren, H. eds., W. de Gruyter, N.Y.), a fact that fits well with the observed unusual domain organization of the *BlmVIII* PKS module (Fig. 4). It is conceivable that the combination of this MT domain with an AT domain specific for a methyl malonate extending unit (Haydock *et al.* (1995) *FEBS Lett.* 374: 246-248) could result in the synthesis of polyketides with a gem-dimethyl moiety via engineering polyketide biosynthesis. Such a gem-dimethyl group has been found to be a very important pharmacophore for the epothilones, a family of hybrid peptide and polyketide metabolites that exhibits a remarkable antitumor activity similar to taxol (Ojima *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96: 4256-4261).

The *BlmIV* and *BlmIII* NRPSs are characterized by the unusual Cy domains as well as the unprecedented Ox domain, providing an efficient biosynthesis for a bithiazole structure. The Cy domain was first defined by Marahiel and co-workers in their study of bacitracin biosynthesis in *B. licheniformis* (Konz *et al.* (1997) *Chem. Biol.* 4: 927-937), and the Cy activity was demonstrated recently by Walsh and co-workers in their study of the

HMWP1 and HMWP2 proteins for yersiniabactin biosynthesis in *Y. pestis* (Gehring *et al.* (1998) *Chem. Biol.* 5: 573-586; Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650).

While thiazoline is the direct product of the Cy domain, the thiazoline-to-thiazole conversion requires an additional oxidation step. We identified at the C-terminus of NRPS-0 an

5 additional domain that shows low, but significant, sequence homology to a family of putative oxidases/dehydrogenases, including the McbC protein of the microcin B17 synthase (Table 1). Microcin B17 synthase catalyzes the synthesis of the oxazole and thiazole-containing peptide antibiotic microcin B17, and McbC has been proposed to play a role in catalyzing the oxazoline/thiazoline-to-oxazole/thiazole conversion (Li *et al.* (1996) *Science* 274: 1188-

10 1193; Milne, *et al.* (1999) *Biochemistry* 38: 4768-4781). Consequently, we propose that this extra domain at the C-terminus of NRPS-0 could provide the oxidase/dehydrogenase activity needed for the biosynthesis of the bithiazole moiety of BLM, defining a novel Ox domain for NRPSs. It is noteworthy that a cell-free preparation from *Sv* ATCC15003 has been reported

15 to catalyze the conversion of phleomycins to BLMs in the presence of NAD⁺ (Takita and Muroka (1990) pages 289-309 in *Biochemistry of Peptide Antibiotics: Recent Advances in the Biotechnology of β -Lactams and Microbial Peptides*, Kleinkauf, H. & von Döhren, H. eds., W. de Gruyter, N.Y.), supporting the hypothesis that the bithiazole moiety of BLM results from stepwise oxidations of a bithiazoline precursor (Fig. 1A). (The phleomycin

20 producer could be imagined to result from the loss of its Ox activity for the first thiazoline ring.) Given the wide distribution of thiazole or oxazole rings in natural products (Ojima *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96: 4256-4261; Li *et al.* (1996) *Science* 274: 1188-1193) exhibiting an impressive array of biological activities, the cloning of the *blmIV,III*

25 genes and the identification of the Ox domain open many opportunities to define the mechanism for thiazole biosynthesis and to potentially synthesize novel thiazole containing molecules by engineering peptide biosynthesis.

Example 2

Identification and characterization of a type II peptidyl carrier protein from the bleomycin producer *Streptomyces verticillus* ATCC 15003.

Results.

Cloning and sequence analysis of the *blmI* gene

In our effort to clone the gene cluster responsible for BLM biosynthesis, we have determined 80 kb DNA sequence from *Sv* ATCC15003 (Fig. 8). Among the orfs identified within the *blm* gene cluster is the small orf of 273 base pairs (bp), *blmI*, which is located approximately 4 kb upstream of the previously characterized *blmAB* resistance locus (Sugiyama *et al.* (1994) *Gene* 151: 11-16; Calcutt and Schmidt (1994) *Gene* 151: 17-21) (Fig. 8B). The *blmI* gene encodes a protein of 90 amino acids with a molecular weight of 9957 and a pI of 6.52 (Fig. 8C). Computer-assisted analysis (Altschul *et al.* (1997) *Nucleic Acids Res.* 25: 3389-3402) of the deduced amino acid sequence indicates that BlmI is very similar to various PCP domains of NRPSs (ranging around 40% identity and 60% similarity, as shown in Figure 9). Like known PCP domains of NRPS, BlmI has the highly conserved signature motif of LGGXS, within which the serine residue is the site for 4'-phosphopantetheinylation (Stachelhaus and Marahiel (1995) *FEMS Microbiol. Lett.* 125: 3-14; Marahiel *et al.* (1997) *Chem. Rev.* 97: 2651-2673). The latter posttranslational modification is generally necessary for peptide biosynthesis; converting the apo-PCP into the functional holo-PCP (Marahiel *et al.* (1997) *Chem. Rev.* 97: 2651-2673; Walsh *et al.* (1997) *Curr. Opin. Chem. Biol.* 1: 309-315). Based on sequence comparison, *BlmI* is most related to PCPs and not to other kinds of carrier proteins that also share the same LGGXS (SEQ ID NO:80) motif and undergo the same posttranslational 4'-phosphopantetheinylation [31], such as the *E. coli* acyl carrier protein (ACP) (Lambalot and Walsh (1995) *J. Biol. Chem.* 270: 24658-24661), the ACP domain of type I PKS and the type II PKS ACP (Cox and Simpson (1997) *FEBS Lett.* 405: 267-272; Carreras *et al.* (1997) *Biochemistry* 36: 11757-11761), the ArCP domain (Gehring *et al.* (1998) *Biochemistry* 37: 2648-2659), and several nodulation related ACP-like proteins (Epple *et al.* (1998) *J. Bacteriol.* 180: 4950-4954; Spaink *et al.* (1991) *Nature* 354: 125-130).

Overexpression of *blmI* in *E. coli*

To overexpress the *blmI* gene in *E. coli*, we directly amplified the *blmI* gene by PCR from the *Sv. ATCC15003* genomic DNA and cloned it into the pQE-60 expression vector to give pBS1 so that BlmI could be produced as a protein with a native N-terminus and a His₆-tag at its C-terminus. However, no production of the BlmI protein was detected, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), upon introduction of pBS1 into *E. coli* M15(pREP4) under the standard overexpression conditions recommended by the manufacturer (Qiagen). We reasoned that the small BlmI protein with its native N-terminus may not be stable in the heterologous host, and hence moved the *blmI* gene from pBS1 into pET-29a to yield the second overexpression construct of pBS2. In the latter construct, BlmI should be produced as a fusion protein with 27 extra amino acid residues at its N-terminus, including an S-tag and the thrombin cleaving site, in addition to the His₆-tag at its C-terminus. Introduction of pBS2 into *E. coli* BL21(DE-3) under the standard overexpression conditions recommended by the manufacturer (Novagen) indeed resulted in overproduction of BlmI. In fact, the bulk of the soluble protein was the overproduced BlmI, which was easily purified by affinity chromatography using Ni-NTA resin (Qiagen). It is noteworthy that fusion of the additional 23 amino acids to the N-terminus of BlmI as in pBS2 and change of the expression system from *E. coli* M15(pREP4) (pBS1) to *E. coli* BL21(DE-3)(pBS2) dramatically improved the expression level of *blmI*.

In vivo 4'-phosphopantetheinylation of the BlmI protein

To establish BlmI as a type II PCP, we tested if it could serve as a substrate for a PCP-specific 4'-PPTase. PPTases catalyze the posttranslational modification of an apo-PCP into a holo-PCP by transferring the 4'-phosphopantetheine moiety from co-enzyme A (CoA) to the conserved serine residue of PCP, and this reaction has been developed recently into a general method to prepare various holo-PCP, holo-ACP, or holo-ArCP from the corresponding apoproteins (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Gehring *et al.* (1998) *Biochemistry* 37: 2648-2659; Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584). Therefore, we decided to investigate the 4'-phosphopantetheinylation of BlmI under both *in vivo* (Ku *et al.* (1997) *Chem. Biol.* 4: 203-207) and *in vitro* (Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650; Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595) conditions.

To examine 4'-phosphopantetheinylation of BlmI in vivo, we chose *E. coli* OG7001 as the expression host, which is a β -alanine auxotroph derived from *E. coli* BL21(DE3) by P1 co-transduction of the *pand* mutation from *E. coli* SJ16 (Epple *et al.* (1998) *J. Bacteriol.* 180: 4950-4954). Upon introduction of pBS2 into *E. coli* OG7001, *blmI* was exceptionally well expressed and the overproduced BlmI protein was readily purified. However, high performance liquid chromatography (HPLC) analysis showed that the purified BlmI was essentially in the apo-form (Fig. 10A), indicative that apo-BlmI was a poor substrate for the *E. coli* endogenous PPTases, such as EntD and ACP synthase (Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Walsh *et al.* (1997) *Curr. Opin. Chem. Biol.* 1: 309-315; Lambalot and Walsh (1995) *J. Biol. Chem.* 270: 24658-24661). To circumvent the poor endogenous PPTase activity, we next co-expressed *blmI* with the *gsp* gene, which was isolated from the gramicidin S producer *Bacillus brevis*, and encoded a PPTase that was known to 4'-phosphopantetheinylate heterologously produced PCPs in *E. coli* (Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Ku *et al.* (1997) *Chem. Biol.* 4: 203-207). We co-transformed pDPT-Gsp, in which the expression of the *gsp* gene was under the control of the T5/Lac promoter (Ku *et al.* (1997) *Chem. Biol.* 4: 203-207), and pBS2 into *E. coli* OG7001. *BlmI* was again very well expressed and the resulting BlmI protein was similarly purified. HPLC analysis showed that at least 60% of overproduced BlmI was modified into the holo-BlmI protein (Fig. 10B). (A PCP domain was similarly 4'-phosphopantetheinylated in vivo before by co-expressing *gsp* in *E. coli* using pDPT-Gsp, and approximately 80% of the PCP was produced in the holo-form (Ku *et al.* (1997) *Chem. Biol.* 4: 203-207).

We next cultured *E. coli* OG7001(pBS2) and *E. coli* OG7001(pBS2/pDPT-Gsp) in the presence of [3-³H]- β -alanine, a known biosynthetic precursor of 4'-phosphopantetheine (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Epple *et al.* (1998) *J. Bacteriol.* 180: 4950-4954). Specific incorporation of [3-³H]- β -alanine into the 4'-phosphopantetheine moiety of holo-BlmI was determined by autoradiographic analysis. Thus, while fermentation of *E. coli* OG7001(pBS2) in the presence of [3-³H]- β -alanine led to an IPTG-dependent overproduction of BlmI, little of the resulting BlmI protein was ³H-labeled, indicative of being produced in the apo-form. In contrast, fermentation of *E. coli* OG7001(pBS2/pDPT-Gsp) in the presence of [3-³H]- β -alanine resulted in a significant increase of IPTG-dependent incorporation of the ³H-label into the overproduced BlmI protein, suggesting a specific incorporation of [3-³H]- β -alanine into holo-BlmI, presumably in the 4'-phosphopantetheine moiety. There were several additional proteins that were also

weakly labeled by [3-³H]-β-alanine. However, both their expression and their incorporation by ³H-label were independent from either IPTG induction or the presence of Gsp, hence these proteins were unrelated to BlmI. (Similar background labeling was reported before for in vivo 4'-phosphopantetheinylation of other PCP (Epple *et al.* (1998) *J. Bacteriol.* 180: 4950-4954)). We also purified the BlmI protein from *E. coli* OG7001(pBS2/pDPT-Gsp) and demonstrated that it was the holo-BlmI protein that was specifically associated with the ³H-activity. Finally, we confirmed the identity of holo-BlmI by subjecting the purified BlmI protein to MALDI-Tof mass spectral analysis (Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584). BlmI produced in the absence of the Gsp PPTase yielded a single peak with a molecular weight of 13,952, suggesting that the produced BlmI protein is in the apo-form (calc., 13,949). In contrast, BlmI produced in the presence of Gsp yielded two species with molecular weight of 13,969 and 14,303, respectively. While the species with the molecular weight of 13,969 represents apo-BlmI, a molecular weight of 14,303 unambiguously confirmed the other protein as holo-BlmI (calc., 14,289). The latter result indicated that the purified BlmI consisted of both the apo- and holo-BlmI proteins, in agreement with the HPLC analysis results (Fig. 10B).

In vitro 4'-phosphopantetheinylation of the BlmI protein

To investigate 4'-phosphopantetheinylation of BlmI in vitro, we chose the Sfp protein as the preferred PPTase, which had been isolated before from the surfactin producer *Bacillus subtilis* (Nakano *et al.* (1992) *Mol. Gen. Genet.* 232: 313-321). (Overexpression of *gsp* in *E. coli* using pDPT-Gsp resulted in predominantly an insoluble Gsp protein (Ku *et al.* (1997) *Chem. Biol.* 4: 203-207). The Sfp PPTase was overproduced in *E. coli* MV1190(pUC8-Sfp) and purified to near homogeneity as described before (Quadri *et al.* (1998) *Biochem.*, 37: 1585-1595; Nakano *et al.* (1992) *Mol. Gen. Genet.*, 232: 313-321). Upon incubation of the purified apo-BlmI with [³H-pantetheine]-CoA in the presence of the Sfp PPTase, we examined the covalent incorporation of the [³H-pantetheine]-4'-phosphopantetheine moiety from CoA into holo-BlmI by autoradiographic analysis. Indeed, the apo-BlmI was quantitatively labeled by [³H-pantetheine]-CoA, and no labeling was observed in the absence of either the apo-BlmI or the Sfp PPTase protein, demonstrating that the Sfp PPTase can recognize apo-BlmI as a substrate and specifically transfer the 4'-phosphopantetheine group from CoA into holo-BlmI.

In vitro aminoacylation of BlmI

Once we established BlmI as a type II PCP that can be readily modified by PCP-specific PPTases into the holo-BlmI protein, we tested if the holo-BlmI could be aminoacylated in trans, requiring an A domain. Since BlmI has no cognate A domain of its own, we turned our attention to another putative biosynthesis gene cluster we have cloned previously from *Sv* ATCC15003, which encodes at least four NRPS and one PKS modules. We have established that this gene cluster is not clustered with the *blm* locus and is unrelated to BLM biosynthesis. From this gene cluster, we amplified by PCR a 1579 bp fragment encoding an A domain, named Val-A, which we predicted to have a molecular weight of 56,581 and a pI of 7.39. We cloned *val-A* into pET-28a to yield pBS3, in which Val-A would be produced as a fusion protein with a His₆-tag at the N-terminus. Introduction of pBS3 into *E. coli* BL21(DE3) under the standard overexpression conditions recommended by the manufacturer (Novagen) resulted in good overproduction of Val-A, predominantly in soluble form, from which Val-A was purified by affinity chromatography using Ni-NTA resin. The purified Val-A protein was active by the amino acid-dependent ATP-PPI exchange assay (Lee and Lipmann (1970) *Method Enzymol.* 43: 585-602; Ku *et al.* (1997) *Chem. Biol.*, 4: 203-207). Among the 23 amino acids tested, Val-A specifically activated valine, an amino acid that is not required for BLM biosynthesis.

To carry out the aminoacylation in trans, we incubated the purified holo-BlmI and Val-A in vitro in the presence *L*-[¹⁴C(U)]valine and ATP (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584). The aminoacylated holo-BlmI-*L*-[¹⁴C(U)]valine species was subjected to SDS-PAGE and specific attachment of *L*-[¹⁴C(U)]valine to holo-BlmI was determined by autoradiographic analysis. Remarkably, the holo-BlmI was specifically labeled by *L*-[¹⁴C(U)]valine in the presence of Val-A, indicative of the formation of the holo-BlmI-S-valine thioester. The in trans aminoacylation between the holo-BlmI and Val-A proteins appeared to be very specific. Neither incubation of *L*-[¹⁴C(U)]valine with Val-A, the apo-BlmI, or the holo-BlmI protein alone, nor incubation of *L*-[¹⁴C(U)]valine with the Val-A and apo-BlmI proteins, resulted in the detection of ¹⁴C-labeled BlmI protein.

Discussion.

Nonribosomal peptides and polyketides are two distinct classes of natural products yet are assembled from amino acids and short carboxylic acids by NRPSs and PKSs, respectively, in strikingly similar strategies (Cane *et al.* (1998) *Science* 282: 63-68).

These fascinating multifunctional enzyme complexes have been classified into two types based on their gene organization and enzyme architecture. Type I enzymes are multifunctional proteins consisting of domains for individual enzyme activities, and type II enzymes are multienzyme complexes consisting of discrete proteins that are largely monofunctional. While both type I and type II PKSs (Fig. 11A and 11C) have been well characterized to account for the vast structural diversities found in polyketide biosynthesis (Hopwood (1997) *Chem. Rev.* 97: 2465-2497), all NRPSs studied so far are exclusively the type I modular enzymes (Fig. 11B) (Kleinkauf and von Döhren: H. (1996) *Eur. J. Biochem.* 236: 335-351; Marahiel *et al.* (1997) *Chem. Rev.* 97: 2651-2673; von Döhren *et al.* (1997) *Chem. Rev.* 97: 2675-2705). It is very tempting to speculate the existence of a type II NRPS that, analogous to type II PKS (Shen and Hutchinson (1993) *Science* 262: 1535-1540; Bao *et al.* (1998) *Biochemistry* 37: 8132-8138; Carreras and Khosla (1998) *Biochemistry* 37: 2084-2088), should consist of discrete proteins possessing enzyme activities such as the A (Stachelhaus and Marahiel (1995) *J. Biol. Chem.* 270: 6163-6169), the PCP (Stein and Morris (1996) *J. Biol. Chem.* 271: 15428-15435), or the C (Stachelhaus *et al.* (1998) *J. Biol. Chem.* 273: 22773-22781) domains of type I NRPSs (Fig. 11D). The fact that both the A (Stachelhaus and Marahiel (1995) *J. Biol. Chem.* 270: 6163-6169; Konz *et al.* (1997) *Chem. Biol.* 4: 927-937; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584; Mootz and Marahiel (1997) *J. Bacteriol.* 179: 6843-6850) and the PCP (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584; Pfeifer *et al.* (1995) *Biochemistry* 34: 7450-7459; Haese *et al.* (1994) *J. Mol. Biol.* 243: 116-122; Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595; Gehring *et al.* (1996) *Chem. Biol.* 4: 17-24; Ku *et al.* (1997) *Chem. Biol.* 4: 203-207) domains of type I NRPSs can act as independent enzymes supports the hypothesis of a type II NRPS.

We have now cloned and sequenced the *blmI* gene, overproduced and characterized the BlmI protein as a bona fide type II PCP, and demonstrated that holo-BlmI can be aminoacylated by a completely unrelated A domain, providing for the first time genetic and biochemical evidence for a type II NRPS enzyme. We concluded BlmI as a type II PCP based on the following criteria. (1) The deduced amino acid sequence of the *blmI* gene is highly homologous to various PCP domains of known NRPSs, in particular at the signature motif of LGGXS within which the 4'-phosphopantetheine prosthetic group is covalently attached to the serine residue (Marahiel *et al.* (1997) *Chem. Rev.* 97: 2651-2673; Stachelhaus and Marahiel (1995) *FEMS Microbiol. Lett.* 125: 3-14). While the current boundaries for a PCP domain in the literature were defined arbitrarily (Stachelhaus *et al.*

(1996) *Chem. Biol.* 3: 913-921) and varied from one PCP to another, we can now re-define a PCP domain for the type I NRPS as a 90 amino acid peptide with approximately 45 amino acids, each flanking the essential serine residue in the LGGXS (SEQ ID NO:81) motif, in light of this discrete BlmI type II PCP (Fig.9). (2) The *blmI* gene has been successfully expressed in *E. coli*, and fusion of a short peptide to the N-terminus of BlmI dramatically improved its overproduction efficiency. While we cannot exclude the effect of different systems on gene expression, i.e., *E. coli* M15(pREP4)(pBS1) vs. *E. coli* BL21(DE-3)(pBS2), we attribute the increase in expression efficiency to the stability of BlmI as an N-terminal fusion protein instead of the otherwise labile BlmI protein with its native N-terminus. Since BlmI was produced predominantly in the apo-form in *E. coli*, apo-BlmI apparently was not a substrate for the endogenous PPTases, such as EntD or ACP synthase, excluding BlmI as an ArCP or ACP, respectively. EntD and ACP synthase are known to 4'-phosphopantetheinylate apo-ArCP and ACP, respectively, to their holo-forms efficiently (Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Walsh *et al.* (1997) *Curr. Opin. Chem. Biol.* 1: 309-315; Lambalot and Walsh (1995) *J. Biol. Chem.* 270: 24658-24661). (3) The apo-BlmI protein serves as a substrate for PCP-specific PPTases that transfer the 4'-phosphopantetheine moiety from CoA to apo-BlmI to yield the holo-BlmI protein. We have demonstrated this posttranslational modification for BlmI *in vivo* with the Gsp PPTase (Ku *et al.* (1997) *Chem. Biol.* 4: 203-207) and *in vitro* with the Sfp PPTase (Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650; Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595), both of which have been extensively used in preparing holo-PCPs. (4) The specific modification of apo-BlmI by 4'-phosphopantetheinylation has been monitored by HPLC analysis (Fig. 10) (Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584) and by specific incorporation of [3-³H]- β -alanine *in vivo* (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Ku *et al.* (1997) *Chem. Biol.* 4: 203-207; Epple *et al.* (1998) *J. Bacteriol.* 180: 4950-4954) and of [³H-pantetheine]-CoA *in vitro* (Gehring *et al.* (1998) *Biochemistry* 37: 11637-11650; Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595), respectively, into the 4'-phosphopantetheine moiety of the holo-BlmI protein. The identity of BlmI was finally confirmed by MALDI-Tof mass spectral analysis that determined the molecular weight for both the apo- and holo-BlmI proteins.

While individual domains of type I NRPSs can function independently and several A (Stachelhaus and Marahiel (1995) *J. Biol. Chem.* 270: 6163-6169; Konz *et al.*

(1997) *Chem. Biol.* 4: 927-937; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584; Mootz and Marahiel (1997) *J. Bacteriol.* 179: 6843-6850) and PCP (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584; Pfeifer *et al.* (1995) *Biochemistry* 34: 7450-7459; Haese *et al.* (1994) *J. Mol. Biol.* 243: 116-122;

5 Lambalot *et al.* (1996) *Chem. Biol.* 3: 923-936; Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595; Gehring *et al.* (1996) *Chem. Biol.* 4: 17-24; Ku *et al.* (1997) *Chem. Biol.* 4: 203-207)

domains have been overproduced, purified, and biochemically characterized, aminoacylation in trans has been successful only between PCPs and their cognate A domains (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584). No

10 aminoacylation between PCP and A domains from different NRPS modules has been observed. These results led to the conclusion that there is a specific protein-protein recognition between the A domain and its cognate PCP (Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584). Such domain-specific aminoacylation, in fact, should be beneficial in maintaining the fidelity of a type I NRPS by providing additional "gating" against

15 misincorporation of non-specifically activated aminoacyl adenylate into the final peptide product. Since a type II PCP such as BlmI lacks its cognate A domain, we asked if BlmI could be aminoacylated by an unrelated A domain of a type I NRPS. Although we have yet to determine the biochemical role of BlmI in vivo, the fact that the *blmI* gene is located in the middle of the *blm* gene cluster suggests that it may be involved in BLM biosynthesis. To

20 avoid the ambiguity of selecting an A domain that may potentially interact with BlmI in vivo, we preferred not to choose any A domain from the *blm* gene cluster to test if it could aminoacylate BlmI in trans. We reasoned that an A domain that is unrelated to BlmI should come from a gene cluster independent from BLM biosynthesis and should activate an amino acid not required by BLM. We chose Val-A because it satisfied both requirements. Val-A is

25 an A domain of a type I NRPS from a gene cluster we have cloned previously from *Sv* ATCC15003 that has proven to be unrelated to BLM biosynthesis, and it specifically activates valine among the 23 amino acids tested. Remarkably, BlmI was efficiently aminoacylated by Val-A. The valine residue is specifically attached in a thioester linkage to the terminal -SH of the 4'-phosphopantetheine moiety of the holo-BlmI protein, as evidenced

30 by the fact that the apo-BlmI was inactive under the identical conditions.

Aminoacylation of holo-BlmI by Val-A represents the first example in which an A domain aminoacylates a protein other than its cognate PCP domain. Since it has been suggested that an A domain of a type I NRPS can transfer the activated aminoacyl adenylate only to its cognate PCP domain because of the specific protein-protein recognition between

the two domains (Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584), the fact that BlmI is aminoacylated by Val-A revealed a distinct feature of a type II PCP. It is very tempting to speculate that type II PCPs such as BlmI may have broad intrinsic substrate specificity toward either the aminoacyl adenylate, the A domain, or both. In fact, the latter feature is reminiscent of the type II PKS ACPs, which have been shown to be interchangeable among different PKS complexes (Shen and Hutchinson (1993) *Science* 262: 1535-1540; Bao *et al.* (1998) *Biochemistry* 37: 8132-8138; Carreras and Khosla (1998) *Biochemistry* 37: 2084-2088). The biosynthesis of *D*-alanyl-lipoteichoic acid in *Bacillus subtilis* (Perego *et al.* (1995) *J. Biol. Chem.* 270: 15598-15606) and *Lactobacillus casei* (Debabov *et al.* (1996) 178: 3869-3876) also involves a discrete ACP-like protein, the *D*-alanyl carrier protein, although the latter clearly is structurally and functionally different from PCPs.

The results strongly suggest the existence of a type II NRPS. In fact, we have already identified within the *blm* gene cluster two additional genes, *blmII* and *blmXI* (Fig. 1B), which encode type II C proteins based on sequence analysis (*see Example 1*).

Significance.

All NRPSs known to date are exclusively the type I modular enzymes that are multifunctional proteins consisting of domains, such as A (Stachelhaus and Marahiel (1995) *J. Biol. Chem.* 270: 6163-6169), PCP (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921), and C (Stachelhaus *et al.* (1998) *J. Biol. Chem.* 273: 22773-22781), for individual enzyme activities (Kleinkauf and von Döhren: H. (1996) *Eur. J. Biochem.* 236: 335-351; Marahiel *et al.* (1997) *Chem. Rev.* 97: 2651-2673; von Döhren *et al.* (1997) *Chem. Rev.* 97: 2675-2705), and control the structural variations of the resulting peptide products by the multiple-carrier thiotemplate mechanism (Cane *et al.* (1998) *Science* 282: 63-68; Stein and Morris (1996) *J. Biol. Chem.* 271: 15428-15435). While individual domains of type I NRPSs can function independently, aminoacylation in trans has been successful only between PCPs and their cognate A domains (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584). We have cloned and sequenced the *blmI* gene, overproduced and characterized the BlmI protein as a bona fide type II PCP, and demonstrated that the holo-BlmI can be aminoacylated by a completely unrelated A domain. Our results provided for the first time the genetic and biochemical evidence to support the hypothesis of a type II NRPS, setting the stage for formulating new research concepts to study peptide biosynthesis. Genetic manipulation of type I NRPS has already been successful in generating novel peptides (Stachelhaus *et al.* (1995) *Science* 269: 69-72). An unprecedented type II NRPS

should shed new light in engineering NRPS proteins, greatly increasing our ability to access peptides with even greater structural diversities.

Materials and methods

General DNA manipulations

5 Plasmids preparation and DNA extraction were carried out by using commercial kits (Qiagen, Santa Clarita, CA), and all other manipulations were carried out according to standard methods (Sambrook *et al.* (1989) *Molecular cloning: a laboratory manual*: (2nd ed): Cold Spring Harbor Laboratory Press: Cold Spring Harbor: USA). *E. coli* strain DH5 α was used as the host for general DNA propagations.

Overexpression of *blmI* in *E. coli* and purification of the BlmI protein

10 The *blmI* gene was amplified from *Sv* ATCC15003 by PCR using a forward primer of 5'-CCG CCC ATG GGT GCT CCG CGT GGC GAG CGG ACC CGG CGC-3' (SEQ ID NO:82, the *NcoI* site is underlined) and a reverse primer of 3'-CCT AGA TCT CCG GTC CCG CTC CCC CGT-5' (SEQ ID NO:83, the *BglII* site is underlined). In order
15 to create the *NcoI* site, the original starting sequence of "ATG AGC" has been changed to "ATG GGT", which resulted in the change of the second amino acid from serine to glycine. The first five codons of *blmI* were also optimized for overexpression in *E. coli*. The PCR-amplified 0.3 kb *NcoI*-*BglII* fragment was cloned into the similar sites of pQE-60 (Qiagen) to form pBS1. Digestion of pBS1 with *NcoI* and *HindIII* and cloning the resulting 0.3 kb
20 *NcoI*-*HindIII* fragment into the same sites of pET-29a (Novagen, Madison, WI) yielded pBS2.

Expressions of *blmI* in *E. coli* M15 (pREP4)(pBS1) and in *E. coli* BL-21(DE-3)(pBS2) and purification of the resulting BlmI protein by affinity chromatography on Ni-
25 NTA resin were carried out under the standard conditions recommended by Qiagen and Novagen, respectively. The incubation temperature was lowered to 30 °C to improve the solubility. The purification of BlmI was monitored by SDS-PAGE on 15% gel. The final pure BlmI protein was desalted on PD-10 column (Sephadex G-25, Pharmacia Biotech, Piscataway, NJ) into 50 mM sodium phosphate buffer, pH 7.8, containing 200 mM NaCl, 10
30 mM MgCl₂, 2 mM dithiothreitol (DTT), 1 mM EDTA, 10% glycerol, and stored at - 80 °C for in vitro assays.

HPLC analysis and MALDI-Tof mass spectral determination

Samples of BlmI (30-70 µg) purified from *E. coli* OG7001(pBS2) or *E. coli* OG7001(pBS2/pDPT-Gsp) were analyzed on a Nova-Pak C18 column (5mm x 10, Waters, Milford, MA) using a Rainin DMAX HPLC unit. The column was developed by a linear
5 gradient of 0-50% acetonitrile in 0.1% trifluoroacetic acid in 25 min, followed by additional 5 min at 50 % acetonitrile, with a flow rate of 0.6 ml/min and detection at 280 nm. MALDI-Tof mass spectral determination was performed on a Bruker Biflex III spectrometer at the Facility for Advanced Instrumentation of University of California, Davis.

In vivo labeling of BlmI with [3-³H]-β-alanine

10 The β-alanine auxotroph *E. coli* strain OG7001 (Epple *et al.* (1998) *J. Bacteriol.* 180: 4950-4954) was transformed with pBS2 and cultured under the same conditions as for *E. coli* BL21(DE3) (Novagen). For co-expression of *blmI* with *gsp*, pDPT-Gsp (Ku *et al.* (1997) *Chem. Biol.* 4: 203-207) was similarly transformed into *E. coli* OG7001(pBS2) and the transformants were cultured in 2xYT (Debabov *et al.* (1996) 178:
15 3869-3876) in the presence of kanamycin (25 µg/ml) and chloramphenicol (50 µg/ml). For in vivo labeling experiment, cells from 2 ml overnight culture of either *E. coli* OG7001(pBS2) or *E. coli* OG7001(pBS2/pDPT-Gsp) were harvested, washed with M9 minimal medium (Debabov *et al.* (1996) 178: 3869-3876), and re-suspended in 2 ml of M9 minimal medium. The latter were used as seed cultures (20 µl) to inoculate 1 ml M9
20 medium with kanamycin (25 µg/ml) or kanamycin (25 µg/ml) and chloramphenicol (50 µg/ml) for *E. coli* OG7001(pBS2) or *E. coli* OG7001(pBS2/pDPT-Gsp), respectively. The resulting culture was incubated at 30 °C, 250 rpm to OD_{600nm} 0.6 and to this was added 10 µCi of [3-³H]-β-alanine (50 Ci/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO) with or without IPTG (1 mM). Total proteins were resolved by SDS-PAGE on 15% gels
25 that were Coomassie blue-stained. To determine ³H-labeling of the overproduced holo-BlmI protein, gels were soaked in Amplifier (Amersham, Arlington Heights, IL) for 20 min, dried between two sheets of cellulose membrane (KOH Development Inc., Ann Arbor, MI), and visualized by autoradiography on X-ray films (Fuji Medical Systems, Stamford, CT).

In vitro labeling of BlmI with [³H-pantetheine]-CoA

30 Expression of *sfp* in *E. coli* MV1190(pUC8-Sfp), purification of the Sfp PPTase to homogeneity, and 4'-phosphopantetheinylation of apo-BlmI by Sfp in vitro were

carried out essentially according to literature procedures (Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595; Nakano *et al.* (1992) *Mol. Gen. Genet.* 232: 313-321). A typical 100 μ l assay solution contained 26 μ M apo-BlmI, 2.9 μ M Sfp, 25 μ M [3 H-pantetheine]-CoA (0.9 μ Ci, 40 Ci/mM), 10 mM MgCl₂, and 5 mM DTT, in 75 mM MES/NaOAc buffer, pH 6.0.

- 5 After 30 min incubation at 37 °C, the assays were stopped by addition of 5 μ l of bovine serum albumin (0.2 mg/ml) and 0.9 ml of cold 10% (v/v) trichloroacetic acid (TCA). The precipitated proteins were collected by centrifugation at 14,000 rpm, 20 min, 4 °C (Eppendorf 5415C centrifuge), washed with 10% TCA three times, and resolved by SDS-PAGE on 15% gel. The 3 H-activity incorporated into holo-BlmI was similarly determined
- 10 by autoradiography as described for in vivo labeling of holo-Blm with [3 H]- β -alanine.

Overexpression of val-A in E. coli and purification and assay of the Val-A protein

- The *val-A* fragment was amplified from Sv ATCC15003 by PCR using a forward primer of 5'-GGA ATT CCA TAT GGG CAC CAC CGT CGC CGC G-3' (SEQ ID NO:84, the *Nde*I site is underlined), and a reverse primer of 3'-GGC AAG CTT GGG ACC GGG CGT GGA GCG C (SEQ ID NO:85, the *Hind*III site is underlined). The PCR-amplified 1.6 kb *Nde*I-*Hind*III fragment was cloned in the similar sites of pET-28a (Qiagen) to yield pBS3. Expression of *val-A* in *E. coli* BL-21(DE-3)(pBS3) and purification of the resulting Val-A protein by affinity chromatography on Ni-NTA resin were carried out under
- 15
- 20 the standard conditions recommended by Novagen.

- Amino acid-dependent ATP-PPi assays were performed essentially according to the literature procedures (Ku *et al.* (1997) *Chem. Biol.* 4: 203-207; Lee and Lipmann (1970) *Method Emzymol.* 43: 585-602). A typical 100 μ l assay solution contained 180 nM Val-A, 1 mM ATP, 0.1 mM PPi with 0.2 μ Ci of 32 P-PPi (11.75 Ci/mmol, NEN Life Science Products, Inc., Boston, MA), 1 mM MgCl₂, 0.1 mM EDTA, and 1 mM L-amino acid in 50 mM sodium phosphate buffer, pH 7.8. After 30 min incubation at 30°C, the assays were stopped by addition of 0.9 ml of cold 1% (w/v) activated charcoal in 3% (v/v) perchloric acid. The precipitates were collected on glass fiber filters (2.4 cm, G-4, Fisher, Pittsburgh, PA), washed successively with 10 ml of 0.2 M sodium phosphate buffer, pH 8.0, 4 ml water,
- 25
- 30 and 1 ml of ethanol, and dried in air. The filters were mixed with 7 ml of scintillation fluid (ScintiSafe Gel, Fisher) and counted on a Beckman LS-6800 scintillation counter to determine the radioactivity.

In vitro aminoacylation of holo-BlmI by Val-A

The aminoacylation of holo-BlmI was carried out essentially according to literature methods (Stachelhaus *et al.* (1996) *Chem. Biol.* 3: 913-921; Weinreb *et al.* (1998) *Biochemistry* 37: 1575-1584). A typical 100 µl assay solution contained 180 nM Val-A, 1.5-
5 2.8 µM apo- or holo-BlmI, 35 µM L-[¹⁴C(U)]-valine (283 mCi/mmol, NEN Life Science Products, Inc., Boston, MA), 5 mM ATP, 10 mM MgCl₂, and 5 mM DTT in 75 mM Tris-HCl buffer, pH 8.0. The reactions were started by the addition of ATP and, after incubation at 37 °C for 30 min, were stopped by addition of 0.9 ml of cold 7% (v/v) TCA. The precipitated proteins were collected by centrifugation at 14,000 rpm, 20 min, 4 °C
10 (Eppendorf 5415C centrifuge) and resolved by SDS-PAGE on a 15% gel. The radioactivity incorporated into the holo-BlmI-L-[¹⁴C(U)]valine species was similarly determined by autoradiography as described for in vivo labeling of holo-BlmI with [3-³H]-β-alanine.

Example 3:

Cloning and characterization of a phosphopantetheinyl transferase from the 15 bleomycin-producing *Streptomyces verticillus* ATCC15003

Multienzymes complexes exist for acyl group activation and transfer reactions in the biogenesis of fatty acids, the polyketide family of natural products (*e.g.* erythromycin, tetracycline), and almost all non-ribosomal peptides (*e.g.* vancomycin, cyclosporin, penicillin). All of these complexes contain one or more small proteins, ~80-100 amino acids
20 long, either as separate subunits or as integrated domains, that function as carrier proteins for the growing acyl chain (acyl-, peptidyl-, and aryl- carrier proteins, abbreviated as ACP, PCP, and ArCP). They are converted from inactive apo-forms to functional holo-forms by the covalent attachment of the 4'-phosphopantetheine moiety of coenzyme A to a conserved serine residue of the carrier-protein substrate. This essential post-translational modification
25 is catalyzed by a family of enzymes known as phosphopantetheinyl transferases (PPTases) (Lambalot *et al.* *Chem. Biol.* (1996) 3:923-936; Walsh *et al.* *Curr. Opin. Chem. Biol.* (1997) 1:309-315).

Research in the field of polyketide and non-ribosomal peptide biosynthesis has been hampered by the inability to fully modify and thus convert to the active form some
30 polyketide synthases (PKS) and polypeptide synthetases (NRPS) when overproduced in heterologous hosts, presumably because the host PPTases are unable to effectively modify these overexpressed protein substrates. Our group is currently involved in the

characterization of the gene cluster responsible for the biosynthesis of the antitumor drug bleomycin in *Streptomyces verticillus* ATCC15003. As bleomycin synthetase is a hybrid NRPS/PKS enzyme, we decided to obtain a PPTase from the producing organism in order to use it *in vitro* or *in vivo* by coexpression with the synthetase genes to produce properly modified, active synthetases for our studies.

Results and Discussion

Cloning of the *pttA* gene from *S. verticillus* ATCC15003.

See 94

The similarities among PPTases from different organisms are reduced to two short motifs separated by 40-45 residues: (V/I)G(V/I)D, and (F/W)(S/C/T)XKE(A/S)hhK (Lambalot et al. *Chem. Biol.* (1996) 3:923-936; Walsh et al. *Curr. Opin. Chem. Biol.* (1997) 1:309-315). Our previous attempts to amplify PPTase sequences from *S. verticillus* chromosomal DNA using degenerate primers according to the two conserved motifs were unsuccessful (unpublished results), so we decided to narrow our target. PPTases have been classified in two groups, according to their specificity for the carrier-protein substrate: PPTases involved in polyketide/fatty acid biosynthesis use acyl carrier proteins (ACPs) as substrate, while those for non-ribosomal peptide biosynthesis use peptidyl carrier proteins (PCPs) or aryl carrier proteins (ArCPs) (Walsh et al. *Curr. Opin. Chem. Biol.* (1997) 1:309-315). Several "NRPS-type" PPTase sequences were used to screen the databases to look for actinomycete homologues, and four proteins of unknown function were found: NshC from *Streptomyces actuosus* (Li et al. *Gene* (1990) 91:9-17), SC5A7.23 from *S. coelicolor* (GenBank AL031107), an unnamed protein from *Streptomyces* sp. strain TH1 (Mori et al. *J. Bacteriol.* (1997) 179:5677-5683), and Rv2794c (later renamed PptT (Quadri et al. *Chem. Biol.* (1998) 5:631-645)) from *Mycobacterium tuberculosis* (GenBank AL008967). The alignment of the actinomycete sequences showed the two motifs conserved in all PPTases and an additional motif - the "THC" motif: PXWPXGX₂GS(M/L)THCXGY (SEQ ID NO:86), located about 15 amino acids upstream of the (V/I)G(V/I)D motif (SEQ ID NO:87). The "THC" motif is not universally conserved in all PPTases, but it can be detected also in some non-actinomycete PPTases like EntD (Coderre et al. *J. Gen. Microbiol.* (1989) 135:3043-3055). Using a recently developed method of PCR primer design (the CODEHOP strategy (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) (Rose et al. *Nucleic Acids Res.* (1998) 26:1628-1635), two primers were designed around the typical C-terminal PPTase motif (primers KEA-1: 5'-T GCA GCA GAA CAG GAG GCK NYC CCA

NKG-3' (SEQ ID NO:88) and KEA-2: 5'-TG GGT CAG CGG GTA CCA NRC YTT RWA-3' (SEQ ID NO: 89, H=C+A, N=A+C+T+G, Y=C+T, K=G+T, R=A+G, W=T+A)), and one primer was designed from the "THC" motif (primer THC: 5'-C GGC ATG GTC GGC TCC HTN ACN CAY TG-3', SEQ ID NO:90, H=C+A, N=A+C+T+G, Y=C+T, K=G+T, R=A+G, W=T+A); this motif is not universally conserved in PPTases of all organisms). Using *S. verticillus* chromosomal DNA as template, no amplification product was detected using the THC and the KEA-1 primers. The set of primers THC/KEA-2 successfully amplified a single band of the expected size (about 250 bp), which was gel-purified and cloned. Eight individual clones were sequenced, and all of them resulted to be identical (except differences due to primer utilization) and highly similar to the putative actinomycete PPTases. The PCR fragment was used as a probe to screen a *S. verticillus* genomic library by colony hybridization. Of the 10,000 colonies screened, 25 positive clones were identified, and then confirmed by Southern analysis to contain the same 4.6-kb *Bam*HI hybridizing band. The 4.6-kb DNA fragment was subcloned, and the nucleotide sequence of a 1,761-bp *Bam*HI-*Sal*I region was determined (SEQ ID NO. 3).

Sequence analysis of the *pptA* locus.

The sequence of the 1,761-bp *Bam*HI-*Sal*I fragment was analyzed for coding regions by using the CODONPREFERENCE and TESTCODE programs of the GCG package (Genetics Computer Group, Madison, Wisconsin). Two complete ORFs (*pptA*, *orf3*) and two incomplete ORFs (*orf1*, *orf4*) were identified within the sequenced region (Figure 13). The first ORF from left to right (designated *orf1*) starts out of the analyzed area and ends with a TGA codon at position 248 of the sequenced fragment. Comparison of the deduced product of *orf1* with proteins in databases showed similarities with Rv2795c from *Mycobacterium tuberculosis* (GenBank AL008967) and SC5A7.22 from *S. coelicolor* (GenBank AL031107), both of unknown function. The second ORF, *pptA*, contains the sequence amplified by PCR and used for the cloning of this locus. It comprises 741 nucleotides, starting with a GTG codon (position 245) which is coupled to the stop codon of *orf1*, and ending with a TAA codon. The starting codon of *pptA* is preceded by a potential ribosomal binding site (RBS), GGGAG. The overall (76.6%) and third codon position (93.9%) G+C contents and the codon usage of *pptA* are similar to those found in other *Streptomyces* genes, with the exception of the stop codon (TAA), which is most uncommon in this group of organisms (Wright et al. *Gene* (1992) 113:55-65). The *pptA* gene encodes a protein of 246 amino acids with a predicted molecular mass of 25,619 Da and a pI of 4.76,

which contains the conserved PPTase motifs. Databases searches with PptA showed significant similarities to the putative actinomycete PPTases (39-52%/48-61% identity/similarity) and to confirmed bacterial PPTases such as EntD from *E. coli* (17%/24% identity/similarity) (Lambalot et al. *Chem. Biol.* (1996) 3:923-936). The third ORF, *orf3*, is separated from *pptA* by an apparently noncoding DNA region of 153 bp, and it is transcribed in opposite and convergent direction with respect to *orf1-pptA*. The gene *orf3* comprises 240 nucleotides, starting with an ATG codon (position 1358) and ending with TGA. The starting codon of *orf3* is preceded by the sequence GAAGG, a potential RBS. The deduced product of *orf3* encodes a protein of 79 amino acids with a predicted mass of 7,555 Da and a pI of 7.17. The Orf3 protein shows similarities to the N-terminal region of SC5H1.35c, a protein of unknown function from *S. coelicolor* (GenBank AL049863). Analysis of Orf3 with the SignalP program (Nielsen et al. *Protein Engineer.* (1997) 10:1-6) predicts an N-terminal signal peptide which would be cleaved between residues 27 and 28 (ALA-DS), suggesting that the mature protein (52 amino acids, 5,099 Da, pI 4.31) would be secreted. Between *orf3* and *orf4* there is an apparently noncoding region of 251 nucleotides. The *orf4* gene is transcribed in opposite and divergent direction with respect to *orf3*. It starts with an ATG codon at position 1610, preceded by a potential RBS (GGAGG), and ends out of the sequenced fragment. The deduced protein product (50 amino acids) of the incomplete *orf4* contains a potential NAD/FAD binding motif, GXGX₂GX₃GX₆G (Scrutton et al. *Nature* (1990) 343:38-43), showing low similarities to diverse oxidoreductases.

Heterologous expression and biochemical characterization of PptA.

In order to test if *pptA* actually encodes a functional PPTase, we decided to overproduce and purify the PptA protein, and assay its catalytic competence on putative substrate proteins or domains. The *pptA* coding sequence was amplified by PCR and cloned into the T5-promoter-based pQE-70 vector, yielding plasmid pQEPPT, in such a way that a hexahistidine tag would be added at the C-terminus of the protein. Expression of the pQEPPT construct in *E. coli* M15(pREP4) resulted in the overproduction of soluble His-tagged PptA which was readily purified by affinity chromatography on Ni-NTA agarose under non-denaturing conditions (FIGURE). Because *pptA* belongs, by sequence similarity, to the subfamily of PPTases involved in nonribosomal peptide synthesis, we first assayed its activity using two different apo-PCPs as protein substrates. The first one, BlmI, has been previously characterized in our laboratory as a discrete peptidyl carrier protein, or type II PCP, whose gene is found within the bleomycin-biosynthesis gene cluster of *S. verticillus*

(Du et al. *Chem. Biol.* (1999) 6:507-517). For the second PCP substrate we used BlmX, a bimodular NRPS protein encoded in the same cluster (Fig. 2), as a source of a type I PCP, i.e. a PCP included in a multidomain NRPS. For the production of this type I PCP, we amplified by PCR a 1,898 bp fragment encoding the adenylation and PCP domains from the second module of BlmX. This DNA fragment was cloned into pMAL-c2x to yield pMAL1617, in which the type I PCP would be produced as a maltose-binding protein (MBP) fusion, MBImX-2, with a predicted molecular mass of 108.5 kDa. Introduction of pMAL1617 in *E. coli* TB1 resulted in good overproduction of MBImX-2, about 40% soluble, which was purified by affinity chromatography using amylose resin. To test the PPTase activity, we incubated the purified PptA with BlmI and MBImX-2 as putative protein substrates in the presence of (³H)-(pantetheinyl)-CoASH, and the tritiated products were subjected to SDS electrophoresis and autoradiography. The well-characterized PPTase Sfp from *B. subtilis*, which exhibits a broad specificity for its protein substrate (Quadri et al. *Biochemistry* (1998) 37:1585-1595), was included as a positive control. In these experiments PptA exhibited a robust phosphopantetheinylation activity on both BlmI and MBImX-2. Having demonstrated that PptA does in fact have PPTase activity on both type I and type II PCP substrates from nonribosomal peptide synthetases, we then proceeded to test two different acyl-carrier proteins (ACPs) as potential substrates. The first one, BlmVIII, is a monomodular multidomain polyketide synthase (PKS) which is encoded in the bleomycin-biosynthesis gene cluster of *S. verticillus* (Fig. 2). BlmVIII contains an ACP domain at its C-terminus, that is a type I ACP. For the second ACP substrate we used TcmM, a type II acyl carrier protein involved in the biosynthesis of the aromatic polyketide tetracenomycin C in *S. glaucescens* (Shen et al. *J. Bacteriol.* (1992) 174:3818-3821; Bao et al. *Biochemistry* (1998) 37: 8132-8138). For the production of TcmM, its coding sequence was transferred from a construct previously made in pET-22b (Gehring et al. *Chem. Biol.* (1997) 4:17-24) into the pET-28a vector to yield pET28a-TcmM, in such a way that a hexahistidine tag should be added at both the N-terminus and the C-terminus of the protein. Plasmid pET28a-TcmM was introduced into *E. coli* BL21(DE3), and TcmM was easily purified by affinity chromatography using Ni-NTA resin. In vitro phosphopantetheinylation assays were performed as before, but using BlmVIII and TcmM as protein substrates, and PptA was able to posttranslationally modified both ACP substrates.

The *pptA* gene is not clustered to the bleomycin-biosynthesis locus.

Some bacterial PPTase genes have been found clustered, or close, to their respective “partner” NRPS genes: *entD* {enterobactin (Coderre et al. *J. Gen. Microbiol.* (1989) 135:3043-3055)}, *sfp* {surfactin (Cosmina et al. *Mol. Microbiol.* (1993) 8:821-831)}, *gsp* {gramicidin (Borchert et al. *J. Bacteriol.* (1994) 176:2458-2462)}, *bli* {bacitracin (Gaidenko et al. *Biotechnologia* (1992) 13-19)}, *lpa-14* {iturin (Huang et al. *J. Ferment. Bioeng.* (1993) 76:445-450)}. To test the possible clustering of *pptA* to the bleomycin-biosynthesis (*blm*) locus, PCR reactions were performed using the THC/KEA-2 primers on several overlapping cosmid clones spanning the *blm* locus plus 30-40 kb upstream and downstream of its putative limits. No amplification product could be obtained in these reactions, showing that the *pptA* gene is not clustered with the *blm* locus.

Discussion

It has been suggested that in organisms containing multiple phosphopantetheine-requiring pathways, each pathway has its own posttranslational modifying activity (Walsh et al. *Curr. Opin. Chem. Biol.* (1997) 1:309-315). Our group has found that *S. verticillus* ATCC15003 contains several PKS and NRPS gene clusters, one of them being responsible for bleomycin production (a hybrid NRPS/PKS system) (Shen et al. *Bioorg. Chem.* (1999) 27:155-171; Du et al. *Chem. Biol.* (1999) 6:507-517). This suggested that the gene encoding the PPTase for the BLM NRPS could be also clustered, or close, to the NRPS genes. However, we have not found this gene after sequencing almost the whole *blm* NRPS locus. Because having this gene could be important for us in order to express functional NRPS modules from the *blm* cluster, we decided to clone the PPTase gene. Additionally, if the “one NRPS cluster - one PPTase” hypothesis was true, it seemed possible to use PPTase sequences as a new kind of probe to clone novel NRPS clusters.

We know that in *S. verticillus* there are several NRPS locus (maybe four), so we expected several “PCP-type” PPTases. However we have amplified only one, and it does not seem to be closely linked to any of the NRPS loci. Interestingly in the actinomycete *Mycobacterium tuberculosis*, whose genome is fully sequenced, there is only one PCP-type PPTase gene, which is not clustered with any of the two NRPS loci present in this organism (Quadri et al, *Chem. Biol.* (1998) 5:631-645). These and other indirect evidences suggest that the idea of cluster-specific PPTases is not the general rule at all but most probably the exception, especially in organisms containing multiple NRPS clusters. And there are strong evidences that at least some PCP-type PPTases can posttranslationally modify PCPs from

different clusters and even different organisms (Quadri et al, *Chem. Biol.* (1998) 5:631-645; Gehring et al, *Biochemistry* (1998) 37:11637-11650). It is most likely that there is only one PCP-type PPTase in *S. verticillus* and that its gene is not necessarily clustered to any of the NRPS loci.

5 Biochemical characterization of the purified PptA protein confirmed not only its PPTase activity but also its broad specificity, comparable to that of Sfp. Different apo-PCPs (type I and type II) and a type-I apo-ACP from the bleomycin synthetase, and the type-II apo-ACP from the tetracenomycin PKS of *Streptomyces glaucescens* were efficiently used as substrates by PptA. These results suggest PptA as a good candidate for heterologous
10 coexpression with NRPS and PKS genes to overproduce active holo-synthase enzymes.

 It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of
15 this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.